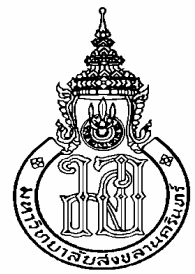

Extraction, Purification and Composition of Bioemulsifier from Spent Yeast Obtained from Thai Traditional Liquor Distillation and Its Application

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ชื่อวิทยานิพนธ์ การสกัด ทำบริสุทธิ์และองค์ประกอบของอิมัลซิไฟด์เออร์ชีวภาพจากยีสต์ที่เหลือจากการกลั่นสุราพื้นบ้านและการประยุกต์ใช้

ผู้เขียน นางสาวปวีณา ดิกิจ

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บทคัดย่อ

การสกัดอิมัลซิไฟด์เออร์ชีวภาพจากยีสต์ที่เหลือจากการกลั่นสุราพื้นบ้านด้วยซิเตรตบัฟเฟอร์ที่เป็นกลางโดยอาศัยความร้อนร่วมกับความดัน อุณหภูมิ 121°ซ, ความดัน 15 ปอนด์ต่อตารางนิ้ว เป็นเวลา 30 นาที ได้ผลผลิตของอิมัลซิไฟด์เออร์ชีวภาพ 0.53 กรัมต่อกรัมน้ำหนักเซลล์แห้ง จากการศึกษาคุณสมบัติทางกายภาพและเคมีของอิมัลซิไฟด์เออร์ชีวภาพเพื่อดูศักยภาพในการใช้เป็นอิมัลซิไฟด์เออร์ในอาหาร พบว่าอิมัลซิไฟด์เออร์ชีวภาพมีกิจกรรมการอิมัลซิไฟด์น้ำมันปาล์มในรูปแบบน้ำมันในน้ำ 60% โดยมีค่าความเข้มข้นน้อยที่สุดที่สามารถคงตัวเป็นอิมัลชันเท่ากับ 20 กรัมต่อลิตร ซึ่งอิมัลซิไฟด์เออร์ชีวภาพที่ได้ประกอบด้วยคาร์โบไฮเดรต 96% และโปรตีน 4% และพบว่าอิมัลซิไฟด์เออร์ชีวภาพมีคุณสมบัติทางอิมัลชันคล้ายกับอิมัลซิไฟด์เออร์ทางการค้า คือ กัมอารบิกและเลซิติน โดยกิจกรรมการอิมัลซิไฟด์น้ำมันปาล์มมีความคงตัวในช่วงพีเอช 3-12, เกลือโซเดียมคลอไรด์ช่วงความเข้มข้น 0-3% (น้ำหนักต่อปริมาตร), แคลเซียมคลอไรด์และแมกนีเซียมคลอไรด์ช่วงความเข้มข้น 0-0.1% (น้ำหนักต่อปริมาตร) นอกจากนี้พบว่าอุณหภูมิ (4°ซ, 10°ซ, อุณหภูมิห้อง, 65°ซ, 100°ซ และ 121°ซ) ไม่มีผลต่อกิจกรรมการอิมัลซิไฟด์ของอิมัลซิไฟด์เออร์ชีวภาพ

การสกัดอิมัลซิไฟด์เออร์ชีวภาพที่มีคุณสมบัติเป็นอิมัลซิไฟด์เออร์จากผนังเซลล์ของยีสต์ *Saccharomyces cerevisiae* KA01 ซึ่งแยกจากน้ำหมักด้วยซิเตรตบัฟเฟอร์ที่พีเอช 7.0 เป็นเวลา 60 นาทีโดยอาศัยการสกัดด้วยความร้อนร่วมกับความดัน อุณหภูมิ 121°ซ, ความดัน 15 ปอนด์ต่อตารางนิ้ว ได้ผลผลิตของอิมัลซิไฟด์เออร์ชีวภาพ 0.64 กรัมต่อกรัมน้ำหนักเซลล์แห้ง อิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้มีคุณสมบัติในการอิมัลซิไฟด์น้ำมันพืชหลายชนิดที่ใช้ในการทดสอบ มีกิจกรรมในการอิมัลซิไฟด์น้ำมันปาล์มในรูปแบบของน้ำมันในน้ำ 65% และมีค่าความเข้มข้นน้อยที่สุดที่สามารถคงตัวเป็นอิมัลชันเท่ากับ 20 กรัมต่อลิตร อิมัลซิไฟด์เออร์ชีวภาพที่ได้มีคุณสมบัติเป็นอิมัลชันเหมือนกับอิมัลซิไฟด์เออร์ทางการค้า คือ กัมอารบิกและ เลซิติน ซึ่งอิมัลชันมีความคงตัวในสภาวะต่างๆได้กว้าง คือ สามารถคงตัวได้ในช่วงพีเอช 5-8, เกลือโซเดียมคลอไรด์ช่วงความ

เข้มข้น 0-3% (น้ำหนักต่อปริมาตร), แคลเซียมคลอไรด์และแมกนีเซียมคลอไรด์ช่วงความเข้มข้น 0-0.1% (น้ำหนักต่อปริมาตร) นอกจากนี้พบว่าอุณหภูมิ (4°ซ, 10°ซ, อุณหภูมิห้อง, 65°ซ, 100°ซ และ 121°ซ) ไม่มีผลต่อกิจกรรมการอิมัลซิไฟด์ของอิมัลซิไฟด์เออร์ชีวภาพ และจากการทดสอบเบื้องต้นพบว่าอิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้จากผนังเซลล์ยีสต์ *S. cerevisiae* KA01 มีศักยภาพในการเป็นอิมัลซิไฟด์เออร์ในการทำน้ำสลัด

จากการศึกษาโครงสร้างของสารอิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้จากผนังเซลล์ยีสต์ที่เหลือจากการกลั่นสุราพื้นบ้านโดยการทำบริสุทธิ์ด้วยคอลัมน์ Sephadex G-100 พบว่าสามารถแยกองค์ประกอบได้ 2 ส่วน ส่วนแรกประกอบด้วยน้ำตาลและโปรตีนซึ่งมีคุณสมบัติเป็นอิมัลซิไฟด์เออร์ ในขณะที่ส่วนที่ 2 มีโปรตีนเป็นองค์ประกอบอย่างเดียวซึ่งไม่มีคุณสมบัติเป็นอิมัลซิไฟด์เออร์จากการศึกษาองค์ประกอบและคุณลักษณะทางเคมีของสารอิมัลซิไฟด์เออร์ชีวภาพพบว่าประกอบด้วยน้ำตาล 96% โดยมีกลูโคสเป็นองค์ประกอบหลักและมีแมนโนสเป็นองค์ประกอบเล็กน้อย และประกอบด้วยโปรตีน 4% มีกรดอะมิโน 17 ชนิดเป็นองค์ประกอบซึ่งมีซีรีนมากที่สุด รองลงมา คือ อะลานีน อิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้ คือโปรตีนที่จับอยู่กับกลูแคน มีน้ำหนักโมเลกุลเฉลี่ย 1.93×10^{5} ดาลตัน ประกอบด้วยคาร์บอน 33.01%, ไฮโดรเจน 5.59%, ไนโตรเจน 0.52% และซัลเฟอร์น้อยกว่า 0.01% การศึกษาหมู่ฟังก์ชันด้วยเทคนิค Fourier Transform Infrared Spectroscopy และ Nuclear Magnetic Resonance Spectroscopy พบว่าอิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้ประกอบด้วยแอลฟา (1⟶4) และเบต้า (1⟶3) กลูแคนจับอยู่กับโปรตีน จากการย่อยด้วยเอนไซม์อะไมเลสและเบต้ากลูคาเนสยืนยันได้ว่าอิมัลซิไฟด์เออร์ชีวภาพประกอบด้วยกลูแคนทั้งสองชนิด

นำสารอิมัลซิไฟด์เออร์ชีวภาพที่สกัดได้มาประยุกต์ใช้เป็นสารอิมัลซิไฟด์เออร์สำหรับการห่อหุ้มเซลล์โปรไบโอติก *Lactobacillus plantarum* D6SM3 โดยศึกษาเปรียบเทียบกับอิมัลซิไฟด์เออร์ทางการค้า คือ กัมอารบิกและทวีน 80 ว่าจะสามารถเพิ่มอัตราการรอดชีวิตของแบคทีเรียโปรไบโอติกที่ถูกห่อหุ้มได้หรือไม่เมื่อผ่านสภาวะที่มีกรดและเกลือน้ำดีในระบบทางเดินอาหาร นอกจากนี้ศึกษาความคงตัวของเซลล์ที่ถูกห่อหุ้มระหว่างการเก็บรักษาเป็นเวลา 28 วันที่ 4°ซ และอุณหภูมิห้อง พบว่าการรอดชีวิตของเซลล์ที่ห่อหุ้มทุกชุดการทดลองเมื่อทดสอบในสภาวะของกรดในกระเพาะอาหารสูงกว่าเซลล์ที่ไม่มีการห่อหุ้มทั้งในสภาวะกรดพีเอช 2.5 และ 3.0 นอกจากนี้มีการศึกษาการรอดชีวิตของเซลล์ที่ไม่ห่อหุ้มและห่อหุ้มโดยระบบต่อเนื่องของกรดและเกลือน้ำดีเลียนแบบสภาวะจริงในระบบทางเดินอาหาร การรอดชีวิตของเซลล์ทั้งที่ไม่ห่อหุ้มและห่อหุ้มลดลงเรื่อยๆ ตลอดระยะเวลาการเก็บรักษาที่อุณหภูมิ 4°ซ ในขณะที่การเก็บรักษาที่อุณหภูมิห้องการรอดชีวิตของเซลล์ทั้งที่ไม่ห่อหุ้มและห่อหุ้มลดลงอย่างรวดเร็ว นอกจากนี้จาก

การศึกษาการกระจายตัวของขนาดด้วยวิธีการกระจายแสงของเซลล์ที่ถูกห่อหุ้มโดยเปรียบเทียบระหว่างการเติมและไม่เติมสารอิมัลซิไฟด์เออร์ พบว่าเซลล์ห่อหุ้มที่เติมสารอิมัลซิไฟด์เออร์สามารถควบคุมขนาดและการกระจายตัวของขนาดเซลล์ห่อหุ้มได้ดีกว่าเซลล์ห่อหุ้มที่ไม่ได้เติมสารอิมัลซิไฟด์เออร์ และนอกจากนี้จากการศึกษาพื้นผิวของเซลล์ที่ห่อหุ้มพบว่าการเติมอิมัลซิไฟด์เออร์มีพื้นผิวเซลล์ที่เรียบกว่าเซลล์ห่อหุ้มที่ไม่มีการเติมอิมัลซิไฟด์เออร์

Thesis Title	Extraction, Purification and Composition of Bioemulsifier from Spent Yeast Obtained from Thai Traditional Liquor Distillation and Its Application
Author	Miss Paweena Dikit
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ABSTRACT

Bioemulsifier from spent yeast was extracted by autoclaving in a citrate buffer pH 7.0 at 121°C under pressure of 15 psi for 30 min. The yield of spent yeast bioemulsifier was 0.53 g/g dry cells weight. The spent yeast bioemulsifier obtained was evaluated for chemical and physical stability to establish its potential use as a natural emulsifier in processed foods. The extracted spent yeast bioemulsifier exhibited emulsion activity of 60% towards palm oil as oil-in-water and had a critical emulsifier concentration of 20 g/l. The composition of the spent yeast bioemulsifier was 96% carbohydrate and 4% protein. The emulsion activity of the spent yeast bioemulsifier towards palm oil was stable over a broad range of pH (3-12), NaCl concentrations of 0-3% (w/v), $CaCl_2$ and $MgCl_2$ concentrations of 0-0.1% (w/v). Temperature of food processing (4°C, 10°C, room temperature, 65°C, 100°C and 121°C) did not affect the emulsion activity of the spent yeast bioemulsifier.

Bioemulsifier from cell walls of *Saccharomyces cerevisiae* KA01 which isolated from sugar palm wine was extracted by autoclaving in a citrate buffer pH 7.0 at 121°C under pressure of 15 psi for 60 min. The yield of yeast bioemulsifier was 0.64 g/g dry cells weight. The extracted yeast bioemulsifier exhibited emulsion with the vegetable oils tested. It showed emulsion activity of 65% toward palm oil as oil-in-water and had a critical emulsifier concentration of 20 g/l. The extracted yeast bioemulsifier had emulsifying properties as good as those of the commonly used food emulsifiers gum arabic and lecithin. Palm oil-in-water emulsion was stabilized over a broad range of conditions, from pH 5 to 8, with up to 3% (w/v) sodium chloride, and up to 0.1% (w/v) $CaCl_2$ and $MgCl_2$ in the aqueous phase. Temperature of food processing (65°C, 100°C and 121°C) did not affect the emulsion activity of yeast

bioemulsifier. Preliminary trials showed that the extracted yeast bioemulsifier from *S. cerevisiae* KA01 had potential for use in salad dressing.

Structure characterization of the bioemulsifier from spent yeast was investigated. Lyophilized sample was fractionated on Sephadex G-100 to yield two fractions coded as fraction I and II. Fraction I was mainly composed of carbohydrates and proteins, showing emulsifying activity whereas fraction II consisted of only proteins and possessed no activity. Composition and chemical characterization of the purified fraction I (the spent yeast bioemulsifier) was then analyzed using various analytical techniques. It was found that the sample contained 96% of carbohydrates consisting mainly of glucose with minor quantities of mannose, and 4% of protein built from 17 amino acids with the highest content of serine followed by alanine. The results also indicated that the sample was protein-bound glucan with the average molecular weight of 1.93×10^5 Da determined by GPC using pullulan as a standard. Elemental analysis (CHNS) showed that the sample contained 33.01% C, 5.59% H, 0.52% N and less than 0.01% S. The functional groups and primary structure of the sample were revealed by FTIR and NMR techniques. The data demonstrated that the sample comprised a mixture of (1→4)-α- and (1→3)-β-D-glucans bound with protein. Enzymatic hydrolysis using α-amylase and β-1,3-glucanase confirmed the presence of both glucans.

The obtained bioemulsifier from spent yeast was applied for encapsulation of probiotic *Lactobacillus plantarum* D6SM3. It was compared with commercially emulsifiers (gum arabic and tween 80) to assess whether it increased the survival rate of encapsulated probiotic lactic acid bacteria when exposed to simulated gastrointestinal conditions. In addition, the stability of the encapsulated cells during storage over 28 days at refrigerated and room temperatures were also evaluated. The survival of all encapsulated cell treatments in simulated gastric juice was higher than free cells at both pH 2.5 and 3.0. The survival of free and microencapsulated cell in simulated small intestinal juice after sequential incubation in simulated gastric juice was also studied. The viability of the free and encapsulated cells showed a gradual decline throughout the storage period at 4˚C but rapidly declined at room temperature. The droplet size distribution of microencapsulated cells was compared between those

with and without an emulsifier by using the Laser diffraction method. The particle size and polydispersity value of microencapsulated cells were controlled better in emulsion with emulsifier added. The surface of encapsulated cells with emulsifier added exhibited smoother characteristics than those without emulsifier.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Emulsifiers are an amphipathic molecules with both hydrophilic and hydrophobic moieties (Vance *et al*., 2003). The hydrophobic and hydrophilic portions are soluble in oil and water, respectively. Hence, the emulsifiers prefer to reside at the interface (Lieberman *et al*., 1988). As a result, emulsifiers cause a reduction of the interfacial tension and allow the two phases to mix more easily. It is used to stabilize the system, and to prevent coalescence of the oil globules (Schramm, 2005). Emulsifiers are widely used in many applications: industrial, agricultural and food processes. Such characteristics confer excellent detergency, emulsifying foaming, and dispersing traits. However, emulsifiers that widely used are chemically synthetic. Although they are very effective in their intended functions, these compounds are gradually losing favor due to increasing pressure from consumers to reduce "artificially" or chemically synthesized additives in food (Shepherd *et al*., 1995). Natural emulsifiers are becoming increasingly more important in the food industry than synthetic ones, which may be potential health hazards for humans (Lukondeh *et al*., 2003). Although some natural emulsifier, plant-derived such as lecithin and gum arabic are already on the market, these emulsifiers have limited use. Lecithin suffers from limited functionality in many food products subjected to modern food processing conditions. Whereas gum arabic, an import from West Africa, is subject to climatic and political unheavals (Shepherd *et al*., 1995).

Nowadays, there are many reports on extracting or isolating and screening of bioemulsifier-producing microorganisms. Because bioemulsifiers have many advantages over the chemically synthesized ones. In Thailand, there is planty of spent *Saccharomyces cerevisiae* yeast that is obtained as a by-product from alcohol fermentation industry. Currently, the Thai government promotes "One Tumbol-One Product, OTOP". Accordingly, one of the OTOP products is a traditional distilled liquor. Generally, producers directly distill palm sugar wine without separation of yeast cells to obtain liquor. After distillation, a huge amount of waste containing yeast

cells is discharged. Spent yeast biomass still remain as waste. Waste disposal is often an environmental problem. Therefore, the possibility of producing high-value-added from by-product is interesting. In addition, it might contribute to improvement of the environmental impact of this technological process.

The inner parts of *S. cerevisiae* cells are isolated and subsequently used as food supplements and flavour enhancers due to their high amount of protein and nucleotides. Whereas the outer parts of the yeast cells such as cell wall remain as waste (Freimund *et al*., 2003). The utilization of yeast cell wall is still limited, being basically used as animal feed (Suphantharika *et al*., 2003). The outer part has received little attention when compared with an inner parts of yeast cells. Recently, β-glucan and mannoprotein that are components of yeast cell wall are becoming attention. Mannoprotein has been shown to be an effective bioemulsifier (Barriga *et al*., 1999; Cameron *et al*., 1988; Torabizadeh *et al*., 1996). β-glucan can be used as a thickening, water-holding, or oil binding agent and emulsifying stabilizer (Thammakitti *et al*., 2004; Worrasinchai *et al*., 2006).

From our study, a bioemulsifier from spent yeast obtained from traditional liquor distillation was extracted and characterized (Dikit *et al*., 2010). Spent yeast from traditional liquor distillation has been suggested as a possible source of income. In addition, it will be help to minimize an environmental impact from the manufacturer. Also, structural characterization of the obtained bioemulsifier was investigated.

Since *S. cerevisiae* is edible and used in the manufacture of food and beverage products, it is assumed that the obtained bioemulsifier would be non-toxic and generally recognized as safe (GRAS) (Cameron *et al*., 1988). Large scale production of this obtained bioemulsifier is possible. It was introduced to emulsion technique of probiotic, *Lactobacillus plantarum* D6SM3 encapsulation. Microencapsulation is a method of improving their viability by retaining probiotics within a polymer membrane or matrix to reduce cell injury or cell loss. Emulsion method is one of the techniques that generate smaller encapsulated particles, when compared with the extrusion method (Krasaekoopt *et al*., 2003). In emulsion technique, emulsifiers are added to form a better emulsion, because the emulsifier lowers the surface tension and results in smaller spheres. This can also provide more

control over the particle size and good polydispersity (Adamson, 1982). Many bioemulsifiers such as gum arabic and several food proteins have been used to get better emulsion in emulsion technique (Kim *et al*., 1996). However, the type of emulsifier has been reported to influence the shape of capsules (Krasaekoopt *et al*., 2003). Therefore, type of bioemulsifier used in emulsion technique should be taken into consideration. The bioemulsifier from spent yeast was compared with commercially emulsifier (gum arabic and tween 80) to assess whether it increased the survival rate of encapsulated probiotic lactic acid bacteria when exposed to simulated gastrointestinal conditions. In addition, the stability of the encapsulated cells during storage over 28 days at refrigerated and room temperatures were also evaluated.

Review of literature

1. Liquor Distillation

A distilled liquor is a liquid preparation containing ethanol purified by distillation of fermented substances such as fruit, vegetables, or grains. Distilled liquor may be classified by the type of raw material used, the process by which the liquor is produced and the country of origin. There are two main carbohydrate sources used in the distilling industry; those that have to be pretreated to yield simple sugars available for yeast fermentation, as in grains, and those that contain fermentable carbohydrates, without further treatment, as in molasses (Bluhm, 1993).

The traditional distilled liquor produced in Southeast Asia are broadly classified into: (1) rice and cereal wine (2) palm wine, and (3) distilled liquor from rice, cereal, or palm wine. Although Southeast Asia lies in the tropical zone, which rich many varieties of fruits, fruit wine could not be found in this area. Palm wine from the sap of palm trees is considered as the fruity wine. Nowadays, the distilled liquor from rice or palm wine is more popular traditional distilled liquor than its origin (rice or palm wine). Rice wine has been switched to distilled liquor of rice or palm wine.

1.1 Processing of distilled liquor production

1.1.1 Preparing grain for fermentation

The first step in most typical process is grain milling, which increases surface area, hence enhances enzymatic activity. The mashing process involves cooking the milled grains in water to hydrate and swell the starch granules. When the slurry is heated, it is referred to as mash. The desired cooking temperature is reached in about two hours. During that time, two basic enzyme reactions occur:

- Proteins are degraded to peptides and amino acids which improves yeast metabolism during fermentation.

- Starch is partly degraded by the enzymes to promote liquefaction. Starch is susceptible to complete enzyme degradation when it has been adequately

heated to cause gelatinization. The temperature required for gelatinization varies with the source of the starch but is always below 100°C (Lee, 1996).

1.1.2 Fermentation

When saccharification is complete, the resulting sugar (largely maltose) are inoculated with a yeast culture, *saccharomyces cerevisiae*. Traditional fermentations are carried out using the microflora or indigenous yeast which are predominantly strains of *S. cerevisiae*. Various yeasts and bacteria participate in natural fermentations. Important non-*Saccharomyces* species are the apiculate yeasts *Kloeckera apiculata* and *Hansensiaspora uvarum* which predominate in the early stages, followed by several other yeasts (e.g. *Candida stellata*, *Torulaspora delbrueckii*, *Kluyveromyces* and *Pichia* spp.) in the middle stages when ethanol levels rise to 3-4% (v/v). The latter stages of fermentation are predominated by *S. cerevisiae* (Fleet and Heard, 1993). The sugar in the mash is usually depleted within 48 h, but to optimize alcohol yields and to obtain characteristic flavors, most fermentation requires 3 days. Most distillers prefer to set fermenters at about 20°C, to control the rate of fermentation so that some activity continues throughout the 72 h period required by regulations. The rate of alcohol production is generally very low in the first 4 to 16 h of fermentation. For the next 24 h, the rate is very rapid and by 48 h of fermentation, 95 to 98% of alcohol will be produced (Walker, 1998). Fermentation is a dynamic process involving a series of reactions:

- Enzymes added after cooking continue to gelatinize starch to dextrins and finally fermentable sugars.

- The yeast metabolized sugars via the Embden-Meyerhof pathway to pyruvate and from there to acetaldehyde and finally to ethanol.

- The lactic acid bacteria inherent in the mash rapidly increase in numbers and produce acid while the rest of the indigenous population dies off.

- The yeast and bacteria produce small amounts of a variety of products, some of which are volatile and contribute to the congeners in the distillate (Fleet and Heard, 1993).

The overall reaction is:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

The moles of ATP are also produced which is used to supply energy for cell maintenance and growth. Theoretically, conversion of a gram of glucose via fermentation yields 0.511 g of ethanol (Reed and Nagodawithana, 1991). This theoretical value is never obtained during fermentation due to carbohydrate utilization for cell maintenance, growth and formation of small amounts of glycerol and higher alcohols (Roehr, 2001). Fermentation efficiency also depend on factors such as yeast strain and environmental parameters. In practice, fermentation efficiencies of slightly more than 90% are obtained. Moreover, the maximum concentration of ethanol that yeast can produce depend upon the yeast strain used. As a general rule, yeast cell growth is inhibited by 10 to 12% (w/v) ethanol while 20% ethanol will terminate cellular metabolism (Reed and Nagodawithana, 1991).

1.1.3 Distillation

The material to be distilled is a mixture of water, ethanol and other compounds of varying volatility. Distillation involves the separation of the components of a solution on the basis of volatility at the boiling point (distillation point). The distillation process based on the different boiling points of water (100°C) and alcohol (78.5°C). The alcohol vapours that arise while the fermented liquid boils are trapped and recondensed to create a liquid of much greater alcoholic strength. (Varnam and Sutherland, 1994).

1.1.4 By-product recovery

Microbial biomass may be dried and utilized as a high protein food or feed supplement. In most distillery, after the removal of alcohol, still bottoms (called whole stillage), are pumped from the distillation column to a dryer house. Whole stillage composed of substances derived from grain, as well as yeast cells and various nutrients formed during the fermentation. Whole stillage may be sold, land applied, sold as liquid feed, or processed and dried to produce distillers dried grains. These dried products are used to fortify dairy, poultry, and swine feeds. Distiller's feeds are rich in proteins (24-35%), fat (8%), various minerals, and other vital growth factors for livestock and poultry (Lee, 1996).

2.Utilization of *Saccharomyces cerevisiae* Biomass from by-Product Sources

2.1 Spent brewer's yeast

Spent brewer's yeast is a by-product of the brewing process. It is inactive yeast (dead yeast cell) remaining after the brewing process generally recognized as safe (GRAS). World-wide spent brewer's yeast is generally sold primarily as inexpensive animal feed which were low price or even used as landfill or dumped into the wastewater treatment plant to produce biogas. Dried yeast is an excellent source of protein for swine and ruminant. (Ferreiro *et al*., 2010). In Thailand, there is plenty of spent brewer's yeast (~ 3000 tons dry basis in 2001 with ~ 10% increase each year) produced as a by-product from breweries. Most of these yeasts are also sold as animal feeds which were low in price (Suphantharika *et al.,* 2003). A lot of yeast waste accumulate every year, has to be disposed with costs. However, its disposal is often an environmental problem. Therefore, the possibility of producing high-value-added products from this by-product. Several attempts have been made to use yeast biomass in biotechnological processes, as raw material for extraction of compounds such as β-glucan. (Ferreiro *et al*., 2010).

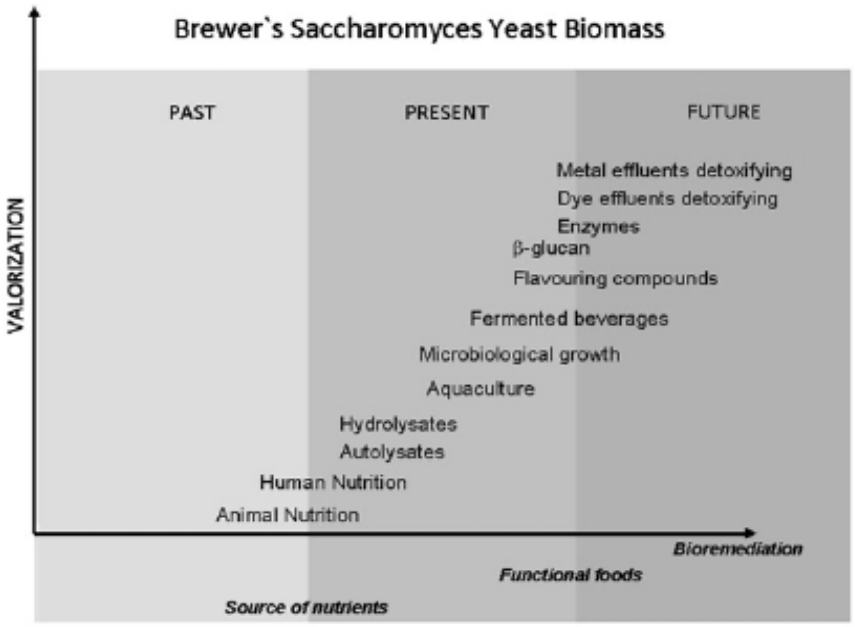


Figure 1. Schematic representation of the brewers yeast biomass applications.
Source: Ferreiro *et al*. (2010).

2.2 Spent distilled yeast

The other by-product source of spent yeast (*S. cerevisiae*) biomass is liquor distillation. It is still underutilized. Yeast disposal is often an environmental problem. Currently, the Thai government promotes “One Tumbol-One Product, OTOP”. One of the OTOP products is a traditional distilled liquor. Generally, producers directly distill palm sugar wine without separation of yeast cells to obtained liquor. After distillation a huge amount of waste containing yeast cells is discharged. Therefore, the possibility of producing high-value-added from by-product are interesting and minimize the environmental impact from their manufacturers.

Yeast cell wall components consisted of mannoprotein, β-glucan and small amount of chitin, which have a potential physiological value. Recently, β-glucan and mannoprotein that are components of yeast cell wall more considerable attention. β-glucan play an important role in the immune system that protects the body against viral, bacterial, and fungal infections (Yadomae and Ohno, 1996 ; Lee *et al*., 2001; Kim *et al*., 2006). Moreover, β-glucan was evaluated for potential food applications (Thammakiti *et al*., 2004). β-glucan was used as a fat replacer in mayonnaise (Worrasinchai *et al*., 2006). Also mannoprotein has been shown to be an effective bioemulsifier (Cameron *et al*., 1988; Torabizadeh *et al*., 1996; Barriga *et al*., 1999). *S. cerevisiae* biomass is the major by-product from wine or brewer’s industry. It was utilized as a source of β-glucan and mannoprotein that used in many applications (Torabizadeh *et al*., 1996; Barriga *et al*., 1999; Suphantharika *et al*., 2003; Thammakiti *et al*., 2004; Worrasinchai *et al*., 2006). Hence, producing a high-value-added product, could benefit breweries and the yeast industry by receiving an additional source of income and eliminating the costs of waste disposal (Jaehrig *et al*., 2007). In addition, It might contribute to improvement of the environmental impact of this technological process.

3. Application from Yeast Cell Wall Component

3.1 Food application

3.1.1 Mannoprotein

Mannoprotein which is a component of the cell wall of yeast can be used as an effective bioemulsifier (Cameron *et al*., 1988; Barriga *et al*., 1999). This bioemulsifier was tested in a mayonnaise formulation. It revealed that mayonnaise with a good quality and appropriate emulsion stability (at least 1 year) could be prepared by using bioemulsifier with a thickener such as carboxymethylcellulose (CMC) (Torabizadeh *et al*., 1996).

Mannoprotein was able to prevent visible wine protein haze formation. White wine clarity is of prime importance for the winemaker as a bottle showing haziness is likely to be rejected by the consumer. The most common form of haze formation in white wine results from the aggregation of grape proteins naturally present in wine (Peatzold *et al*., 1990; Water *et al.* 1991). To prevent haze formation, winemakers usually lower the concentration of wine proteins through the use of bentonite. Bentonite also results in the costly loss of wine in lees and removes wine aroma components, hence lower wine quality. *S. cerevisiae* yeast mannoprotein could protect wine from protein haze spoilage (Water *et al*., 1994; Dupin *et al*., 2000; Caridi, 2006). The presence of yeast mannoprotein in wines has many consequences: (i) reduction of the visible protein haze in white wine (Dupin *et al*., 2000; Water *et al*., 1994) (ii) increase in colour stability, (iii) inhibition of tannin aggregation, (iv) stimulation of malolactic fermentation, (v) protection of wine from tartaric acid precipitation, and (vi) interaction with wine volatile compounds (Chalier *et al.,* 2007). It is known that aroma compounds are able to interact with polysaccharides and proteins. Mannoprotein is potentially change the sensory properties of wine. Lubber *et al*. (1994) studied the interaction of mannoproteins with wine aroma compounds. The strength of interaction was found to depend on the nature of the aroma compound and on the nature of macromolecule. The interaction between mannoproteins and aromatic compounds can lead to modifications of volatility and aromatic intensity of wines. When wine is aged on its lees with no fining, mannoproteins are present and are free to interact and to fortity the existing aroma components.

3.1.2 Glucan

A preparation of β-glucan, obtained from spent brewer's yeast, was evaluated for potential food applications. It was found that it had higher apparent viscosity, water-holding capacity and emulsion stabilizing capacity. These finding suggest that β-glucan can be use in food products as a thickening, water-holding, or oil-binding agent and emulsifying stabilizer (Thammakiti *et al*., 2004). In addition, β-glucan is not hydrolyzed in the human digestive tract, and hence function as a noncaloric food (Temelli and Burkus, 2000). Morever, Spent brewer's yeast β-glucan can be used as a fat replacer in mayonnaise (Worrasinchai *et al*., 2006). β-glucan partially substitute vegetable oil levels in mayonnaise. The substitution levels of not more than 50% of oil used were found to be acceptable. There were two advantages of using β-glucan for reducing fat in mayonnaise formulation. First, decreasing the calorie content of the emulsions, because of a lower proportion of oil in the formulation. Second, utilizing products derived from industrial by-products. This type of mayonnaise is suitable for health conscious people who are concerned about their fat intake and who are on a diet.

3.2 Bioactive and medicinal applications

β-glucan has attracted attention because of its bioactive and medicinal properties as immune-stimulating (Vetvicka *et al*., 2008), anti-microbial, anti-infective, anti-viral, wound-healing (Bohn and BeMiller, 1995), anti-tumoral (Wang *et al*., 2004; Magnani et al., 2009) and cholesterol-lowering (Volikakis *et al*., 2004). β-glucan is known as a potent immunostimulant and has significant augmenting effects on the host defense system (Yadomae and Ohno, 1996). Glucan from spent brewer's yeast significantly ($p \leq 0.05$) enhanced phenoloxidase (PO) activity of black tiger shrimp hemolymph as compared to controls without added glucan (Suphantharika *et al*., 2003). Besides, β-glucan is known to process antimicrobial and antitumor activities by enhancing host immune function, and activating macrophages, neutrophils and NK cells by binding to the β-glucan receptor on these cells (Bohn and BeMiller, 1995). β-glucan is a promising candidate as a immune-stimulatory agent for immune-compromised patients or those who are infected by multidrug-resistant bacteria (Yoshida *et al*., 1996). At present, most anticancer drugs exhibit serious the

side effect of myelo suppression which can lead to fatal microbial infection and sepsis. β-glucan may also serve as an ideal adjuvant for these anticancer drugs since it stimulates the immune function (Lee *et al*., 2001).

4. Yeast Biomass-Derived Products

Yeasts are unicellular fungi and are used for baking and ethanol production for thousands of years. Nowadays, the worldwide production exceeds 2.5 million tons. Some yeast factories grow not only yeast for bakeries, but also produce so-called yeast extracts, which are obtained after mechanically or enzymatically supported autolysis. The inner parts of the cells are isolated and subsequently used as food supplementely and flavor enhancers due to their high amounts of proteins and nucleotides. The outer parts of the yeast cells, the cell walls, remains as waste for which so far no commercially use has been established except as a supplement for animal feed. Hence, the cell walls are therefore as raw material for the manufacture of glucan and mannoprotein They are cheap and show a sufficiently high content of glucan and mannoprotein (Freimund *et al*., 2003).

Yeast biomass, mainly in the form of baker's yeast, represents the largest bulk production of any single-cell microorganism thoughout the world. Several million tons of fresh *S. cerevisiae* cells are produced annually for human food use. Due to the expansion of the brewing industry, the brewers then began to experience the problem of excess yeast disposal, which accounted for a substantial proportion of their waste load. As a means of disposal, the spent yeast was often sprayed on agricultural lands as a source of fertilizer (Reed and Nagodawithana, 1991). In modern times, many other applications of yeast cells and yeast cell extracts have emerged (Table 1). Most yeast biomass for industrial use is derived from *S. cerevisiae* (Walker, 1998).

Table 1. Industrial uses of yeast biomass.

	Type of yeast product	Examples of uses
Whole-cell products	1.Active dried yeast	Baking, brewing, winemaking and distilling
	2. Fodder yeast/ single-cell protein	Animal feed
	3. Biosorbent yeasts	Heavy metal sequestration
	4. Bioligical control yeast	Antifungal agents in agriculture
Extracted-cell products	1. Yeast extracts	Food use and microbiological Growth media
	2. Yeast RNA derivatives	Flavour enhancers and pharmaceutical use
	3. Yeast cell walls	Food and pharmaceutical use
	4. Yeast enzymes	Invertase and lactase for food use

Source: Walker (1998)

4.1 Whole-cell products

- Animal feed

Yeast biomass in its inactive dried form is used widely as a feed supplement. It is used in poultry and pig starter feeds (Jaehrig *et al*., 2007). The use of brewer's and distiller's by-product yeast in feed has already been mentioned. In countries that lack cheap sources of oil seed meals (mainly soybean meal), *Candida utilis* biomass is used extensively as a protein supplement. Live yeast cells in the form of active dried yeast or yeast culture are also used in the feed industry (Chaucheyras-Durand *et al.,* 2008). Active dry yeast was used as a supplement to ruminant feeds at levels that efficiency in cattle. Besides, whole-cell yeast biomass can applied as livestock growth factor in ruminants. Live cultures of *S. cerevisiae* stabilized the rumen environment and improved nutrient availability to increase animal growth or milk yields (Stella *et al.,* 2007). The yeasts may be acting to scavenge O_2 and prevent

oxidative stress to rumen bacteria, or they may provide malic and other dicarboxylic acids to stimulate rumen bacteria growth (Walker, 1998).

4.2 Extracted-cell products

Yeast cell walls represent by-products from yeast extract. This material has potential uses in food industry as a stabilizing agent. Cell wall mannoprotein of *S. cerevisiae* has applications as a bioemulsifier in food products (Torabizadeh *et al*., 1996). Bohn and BeMiller (1995) have also discussed the utility of yeast cell wall glucans in enhancing the human immune system. These immunomodulating properties are associated with the 1,3-β-D-glucan moieties of the *S. cerevisiae* cell wall.

- Immune system

In *S. cerevisiae*, the two major polysaccharides, constituting up to 90% of cell wall dry weight, which have remarkable properties to interact with the immune system of the host. Modulation of mucosal immunity by the binding of these two polysaccharides to specific receptors of immune cells provides beneficial effects on animal health and resistance to diseases. Interestingly, both types of the major polysaccharide constituents of the yeast cell wall-β-D-glucans and α-D-mannans have been recently recognized to be capable of pronounced modulation of the immune system of various living organisms from insects to human through specific interactions with different immuno component cells (Medzhitov and Janeway, 2000).

The two commercial preparations Bio-Mos® and MTB 100® are produced from the yeast cell wall of *S. cerevisiae* by Alltech Inc. (Nicholasville, KY). Due to the content of the active polysaccharides β-D-glucan and α-D-mannans, application of these two products as feed supplement to pig led to the beneficial results such as enhanced weanling piglets protection from bacterial infections and increased weight gain (Kim *et al*., 2000; Kogan and Kocher, 2007).

5. Composition of Yeast Cell Wall

The cell wall is an organelle to yeasts localized at the interface between the microorganisms and the environment. The yeast cell wall has been studied extensively in *S. cerevisiae* and in the human fungal pathogens *Candida albicans* (Firon *et al*., 2004). The yeast cell wall is essential for maintaining the osmotic balance of the cell, for creating and maintaining the shape of the cell, and for morphogenesis (Groot *et al*., 2005). Cell wall represented 26–32% of the cell dry weight, were prepared from several strains of the yeasts *Kloeckera apiculata, Debaryomyces hansenii, Zygosaccharomyces bailii, Kluyveromyces marxianus* and *S. cerevisiae* (Table 2). The cell walls of these yeasts were composed of 84-89% carbohydrate and 11-16% protein (Nguyen *et al*., 1998).

Table 2. Cell wall content and composition of several yeast species.

Yeast species	Cell wall[a](%)	Cell wall composition(%) ± SD	
		Polysaccharides	Protein
Kloeckera apiculata	29.8 ± 1.8	86.8 ± 1.9	13.2 ± 0.6
KI. apiculata	28.7 ± 0.9	86.1 ± 2.2	13.9 ± 0.6
Debaryomyces hansenii	32.0 ± 2.0	89.1 ± 2.4	10.9 ± 0.4
D. hansenii	29.9 ± 1.5	86.1 ± 1.9	15.9 ± 0.7
Zygosaccharomyces bailii	25.8 ± 1.6	85.1 ± 1.7	14.9 ± 0.6
Z. bailii	27.1 ± 0.7	83.8 ± 2.0	16.2 ± 0.6
Kluyveromyces marxianus	29.5 ± 1.4	86.8 ± 2.1	13.2 ± 0.5
Kluy. marxianus	32.5 ± 1.7	88.6 ± 2.7	11.4 ± 0.4
Saccharomyces cerevisiae	29.0 ± 1.6	86.5 ± 2.5	13.5 ± 0.5

[a] Based on cell dry weight.

Source: Nguyen *et al*. (1998)

The cell wall of *S. cerevisiae* is organized into two layers that are made up of only four classes of macromolecules, namely mannoprotein, β-1,6-glucan, β-1,3-glucan and chitin (Fig. 2). These components are all interconnected by covalent bonds (Kapteyn *et al*., 1999). Outer layer consists of mannoprotein which are extensively *O*

and *N* glycolsylated. Inner layer consists of β-1,3-glucan, β-1,6-glucan and chitin (Table 3). The ratio of these compounds are strongly dependent on the conditions during growth and processing of the yeast (Freimund *et al*., 2003). They are densely packed and limit wall permeability to solutes (Lipke and Ovalle, 1998).

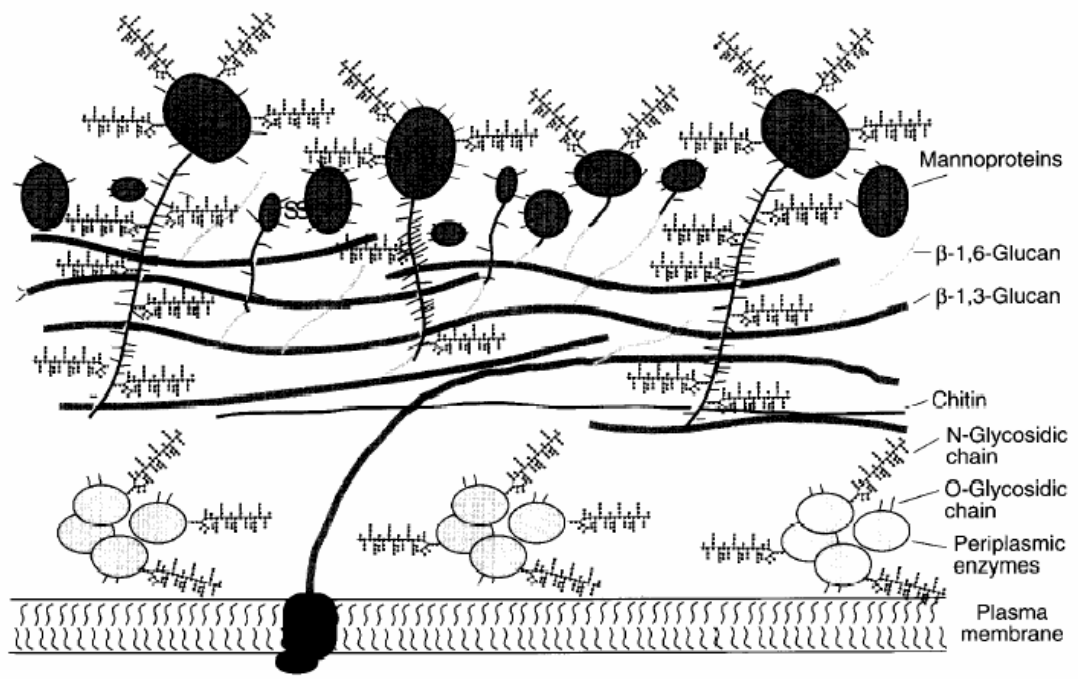


Figure 2. Composition and structure of the cell wall of *Saccharomyces cerevisiae*. Source: Osami (1997)

Table 3. Major components of *Saccharomyces cerevisiae* cell walls.

Component (degree of polymerization)	Mean molecular mass (kDa)	% of cell wall mass	Relative molar ratio
β-1,3 glucan (1500)	240	50	1.0
β-1,6 glucan (150)	24	10	2
Mannoprotein	100-200	40	1.2-2.4
Chitin (120)	25	1-3	0.1-0.3

Source: Lipke and Ovalle (1998)

5.1 Mannoprotein

Mannoprotein was extracted from the cell wall of *S. cerevisiae* is an effective bioemulsifier and is freely soluble in water (Cameron *et al*., 1988). The structural polymer of cell wall mannoprotein consists of a minor part of proteins, which is covalently linked to the major part of a mannan polymer (Vinogradov *et al*., 1998). It contains approximately 90 % mannose and 5-10% protein. Structural mannoproteins are interspersed within a network of glucan to form the outer layer of *S. cerevisiae* cell wall (Cameron *et al.,* 1988). It has two types of mannan chains are attached. One of these carbohydrate polymer is a long, branched, bulky mannan with 40-100 mannose units. It's made up of α-1,6-glycosidic mannose and numerous α -1,2 and α -1,3 side chains. These longer polysaccharides are attached to the protein by *N*-linkages at asparagine residues and the side chains may be phosphorylated. The second type of mannan chains are only 1 to 5 mannose units long and not phosphorylated. These short mannan chains are attached to the protein by *O*-linkages to serine or threonine (Barriga *et al*., 1999) (Fig. 3). Since *S. cerevisiae* is edible and is used in the manufacture of food and beverage products, it is expected that a mannoprotein bioemulsifier would be nontoxic and generally recognized as safe (GRAS) (Cameron *et al*., 1988).

The synthesis of *O*-linkages residues starts in the endoplasmic reticulum with the transfer of the first mannose to Ser/Thr residues and continues in the golgi apparatus to end up with linear molecules of 1 to 5 mannoses. Synthesis of *N*-linkages oligosaccharides also starts in the endoplasmic reticulum membrane with the construction of the lipid-linked core structure and subsequent transfer to the protein. The core structure is further elongated and branched in the golgi apparatus with the addition of mannose units and mannosyl phosphate groups (Corbacho *et al.,* 2005).

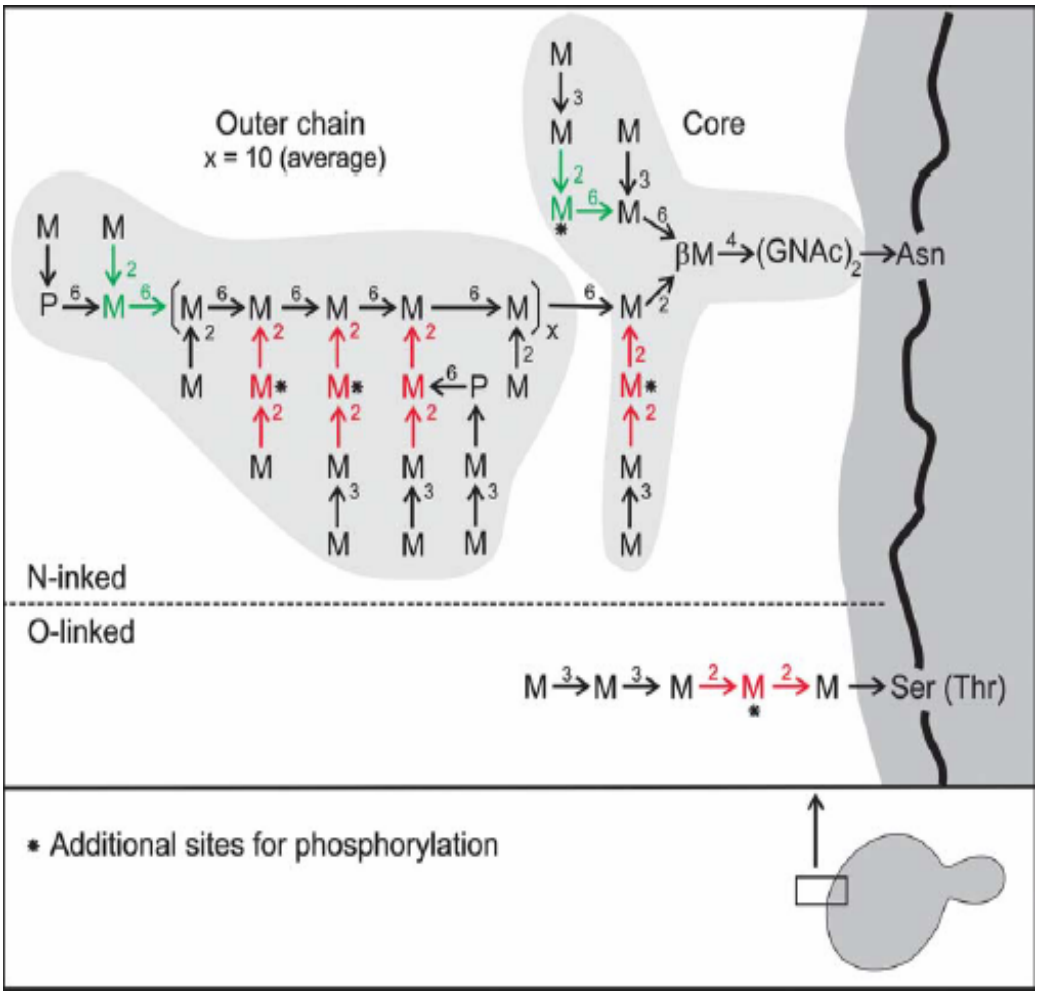


Figure 3. Structure of the *S. cerevisiae N*- and *O*-linkages oligosaccharides in the cell wall.

Source: Corbacho *et al.* (2005)

5.2 Glucan

The cell wall glucans are composed of a 1,3-glycosidic backbone and 1,6-glycosidic intermediate chains. Together they form a three-dimensional network. β-glucan is generally described as a polymer of glucose that widespread in many bacteria, yeasts, molds, mushrooms, algae and higher plants such as oat and barley. The most important of β -glucan is cell wall of yeast *S. cerevisiae* (Klis *et al.*, 2002). There are two different glucans in the cell wall of yeast.

5.2.1 β-1,3 glucan

β-1,3 glucan is the main structural component together with chitin. It is responsible for the rigidity of the cell wall thus determining its shape and strength. It is predominantly a β-1,3-linked linear molecule consisting of about 1500 glucose residues and containing 40-50 glucose residues involved in branching through their C_6-atom (Kapteyn *et al.,* 1999). Consequently, β-1,3 glucan has multiple non-reducing ends allowing it to function as acceptor for both β-1,6 glucan and chitin. When chitin is attached to β-1,3 glucan, β-1,3 glucan becomes insoluble in alkali, explaining the presence of alkali-soluble and alkali-insoluble β-1,3 glucan in the cell wall (Kollar *et al.*, 1997).

5.2.2 β-1,6 glucan

β-1,6-glucan is an essential fungal-specific component of the *Saccharomyces cerevisiae* cell wall that interconnects all other wall component into a lattice (Shahinian and Bussey, 2000). It is a highly branched polysaccharide that has small molecules consisting of approximately 140 glucose residues, and representing only about 5% of the cell wall (Kapteyn *et al*., 1999). It attach mannoprotein via their glycosylphosphatidylinositol (GPI) remnant to β-1,3 glucan and chitin. In addition, it may function as acceptor site for chitin, particularly in case of cell wall stress (Klis *et al.,* 2002).

5.3 Chitin

Chitin is a polymer of *N*- acetylglucosamine, forms insoluble fibril network in the cell wall. It account for only 1-2 % of the cell wall. Part of the chitin is located in a ring at the base of the bud. However, some chitin is also deposited, in a dispersed fashion and in the lateral walls (Kapteyn *et al*., 1999). Chitin is glycosidically linked to non-reducing branches of the β-1,3 glucan and β-1,6-glucan (Lipke and Ovalle, 1998).

6. Factors Affecting Mannoprotein and Glucan Structures

6.1 Yeast strains and cultivation conditions

Nguyen *et al.* (1998) compared composition of the cell walls of several yeast strains including *K. apiculata*, *D. hansenii*, *Z. bailii*, *K. marxianus* and *S. cerevisiae*. They found that proportions of different fractions of cell walls varied with yeast species and strains. Yeast cell wall consists of about 85-90% polysaccharides and 10-15% protein. The polysaccharide component consists of a mixture of mannan, glucan and small amounts of chitin. Most of the protein is covalently linked to the mannan, which described as mannoprotein. Mannoprotein comprised between 25% and 34% of the walls. It mostly consisted of mannose (89-96%) and a small percentage of glucose (1-7%) depending on the yeast strains. These fractions also contained a small amount of glucosamine (1-6%). The N content of this wall fraction varied with the yeast species. *D. hansenii* had a lower N content (about 2.5%) than *Z. bailii* (4%) (Tables 4 and 5). Whilst, the content of alkali-insoluble glucan ranged from 15- 48% and the content of alkali-soluble glucan range from 10 - 48%.

The cell wall is a dynamic structure that can adapt to physiological (i.e. from logarithmic to stationary phase) and morphological changes (conjugation, sporulation or pseudohyphal growth). The variability of the cell wall compositions depend on several growth conditions, including the mode of cultivation, the nature of the carbon source, temperature, pH and aeration. Aguilar-Uscanga and Francois (2003) found that the dry mass and polysaccharides content of the cell wall could vary with the nature of the carbon source, nitrogen limitation, pH, temperature, aeration and with the mode of cell cultivation (shake flasks and controlled fermentors). Growth parameters like pH and oxygen availability of shaking flasks are fixed at the start of the culture and greatly vary with the progression of growth, while in controlled batch reactors, these parameters can be kept constant throughout the fermentation process. The variable proportion of β-glucan and mannan more noticeable in yeast cells cultivated in shake flasks than in batch fermentors.

Table 4. Yields of wall components from several yeast species.

Yeast species	Yield (%)			
	Glucan		Chitin	Mannoprotein
	Alkali Soluble	Alkali insoluble		
- *Kloeckera apiculata* 2164	43.8 ± 0.3[*1]	15.5 ± 3.2[*3]	0.47 ± 0.05	29.3 ± 4.4
- *Kl. apiculata* 2168	38.1 ± 5.5	17.7 ± 2.1[*3]	0.18 ± 0.02[*5]	30.2 ± 3.7
- *Debaryomyces hansenii* 2577	14.5 ± 3.2[*2]	48.4 ± 3.2[*4]	7.74 ± 0.55[*6]	28.9 ± 2.6
- *D. hansenii* 1570	37.4 ± 1.9	20.9 ± 2.8	2.10 ± 0.19[*7]	32.3 ± 1.2
- *Zygosaccharomyces bailii* 1299	37.3 ± 4.1	23.8 ± 0.6	0.71 ± 0.05	29.0 ± 5.7
- *Z. bailii* 3704	33.9 ± 2.0	20.8 ± 3.9	0.64 ± 0.07	34.5 ± 2.6
- *Kluyveromyces marxianus* R157	10.0 ± 4.2[*2]	40.6 ± 5.4[*4]	4.06 ± 0.35[*8]	34.1 ± 4.1
- *Kluy. marxianus* 1586	48.0 ± 3.1[*1]	18.5 ± 1.2[*3]	1.11 ± 0.10	25.6 ± 3.5[*9]
- *Saccharomyces cerevisiae* 1117	33.5 ± 4.1	37.3 ± 3.0	3.36 ± 0.25[*8]	24.4 ± 3.5[*9]

[*1-*9] Mean values within the same column followed by different superscript were significantly different ($p<0.05$) from those without a superscript

Source: Nguyen *et al.* (1998)

Table 5. Monosaccharide composition and nitrogen content of wall components of several yeast species. The monosaccharide composition is shown as glucose: mannose: *N*-acetylglucosamine.

Yeast species	Mannoprotein	
	Monosaccharide	N(%)
Kl. apiculata 2164	6:92:2	3.2
Kl. apiculata 2168	7:89:4	3.6
D. hansenii 2577	4:90:6	2.5
D. hansenii 1570	3:96:1	2.5
Z. bailii 1299	5:94:1	4.3
Z. bailii 3704	4:94:2	3.9
Kluy. marxianus R157	1:95:4	3.5
Kluy. marxianus 1586	6:93:1	3.8
S. cerevisiae 1117	5:93:2	3.0

Source: Nguyen *et al.* (1998)

6.2 Extraction methods

There are currently two methods used to isolate and to purify mannoproteins: the enzymatic method and the heat-treatment method. Whereas, traditional method for preparing β-D-glucans was based on repeated extraction with acid or alkali solutions (Williams *et al.,* 1992).The enzymatic method uses β-1,3-glucanase to decompose yeast cell wall and to liberate the desired mannoproteins. The other method uses high temperature to release mannoproteins from yeast cell wall by using bufferd pH 7. Mannoproteins extracted by high temperature method are richer in proteins than those extracted by enzymatic method, but are similar in most other respects (Feuillat, 2002).

6.2.1 Extraction in neutral condition

Mannoprotein emulsifier was extracted from whole cells of bakers' yeast by autoclaving in neutral citrate buffer at 121°C for 3 h (Cameron *et al.,* 1998). It was found that this procedure had been shown to release an emulsifying agent. Crude emulsifier from bakers' yeast by heat extraction contained approximately 17%

carbohydrate and 54% protein. The heat-extraction procedure is expected to solubilize the structural mannoprotein in the outer layer of the cell wall.

Freimund *et al*. (2003) have developed a new non-degrading process for the isolation of glucan from yeast cell walls comprising gentle extraction steps and enzymatic treatments without any extreme pH values, combining gentle extractions by hot water and organic solvents with enzymatic treatments. The resulting glucan was obtained in high yields and with high purity, and mannoprotein, also obtained as a byproduct. The extraction of water-soluble compounds from biological materials with hot water is approved procedure for the isolation of valuable natural products. They used this step to remove water-soluble components of the yeast cell walls, i.e. particularly mannoprotein, other proteins and pustulan. Yeast cell wall in H_2O was adjusted to pH 7 with NaOH. It was found that the precipitated compounds consist of about 14-15% of protein and 81-83% of carbohydrates, mainly composed of mannose 94% besides a small amount of glucose (6%). The insoluble residue after hot water extraction and protease treatment contained in principle only glucan and lipids. There were two possibilities for removal of the lipids and obtaining the desired glucan. First, sediment can be washed with an excess of acetone, filtered and dried. Second, spray-drying of the sediment and subsequent extraction with ethanol (Fig. 4).

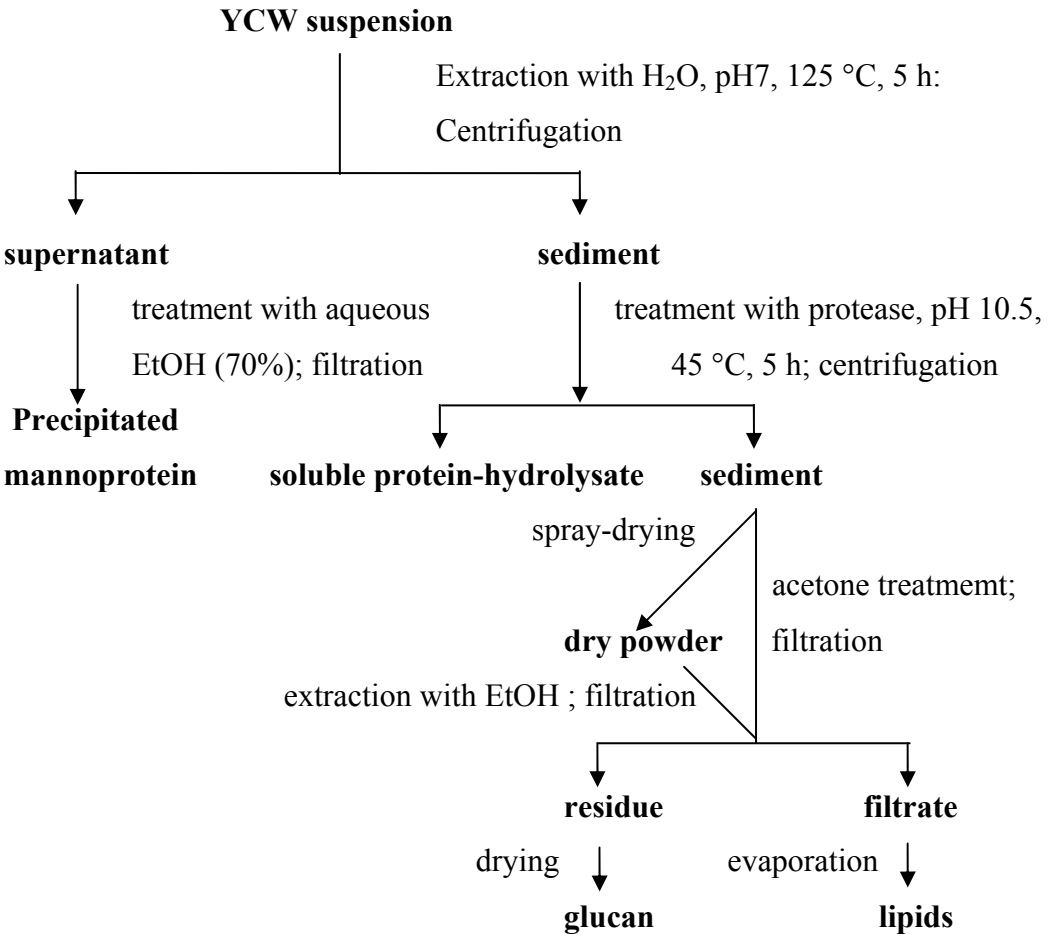


Figure 4. Schematic process for the fractionation of yeast cell wall (YCW).
Source: Freimund *et al*. (2003)

6.2.2 Extraction in alkali condition

Hot sodium or potassium hydroxide has been proven to be very efficient to remove proteins and alkali-soluble polysaccharides from yeast cell walls. Numerous papers and patents are based on the treatment of yeast or yeast cell walls with hot NaOH or KOH, preferred at concentrations of 0.75-1.0 M and temperatures of 60-100˚C. Nguyen *et al*. (1998) studied extraction of the walls with potassium hydroxide at 4°C followed by saturation of the alkali-soluble extract with ammonium sulphate gave fractions of mannoprotein alkali-soluble glucan and alkali-insoluble glucan, chitin was associated with the alkali-insoluble glucan (Fig.5). It was found that *S. cerevisiae* composed of mannoprotein which the ratio of glucose : mannose : N-acetylglucosamine 5:93:2 and protein (N%) 3%. Whereas, alkali-soluble glucan

composed of the ratio 90:10:0 and protein (N%) 2.1%, alkali-insoluble glucan 89:2:9 and protein (N%) 1.5%.

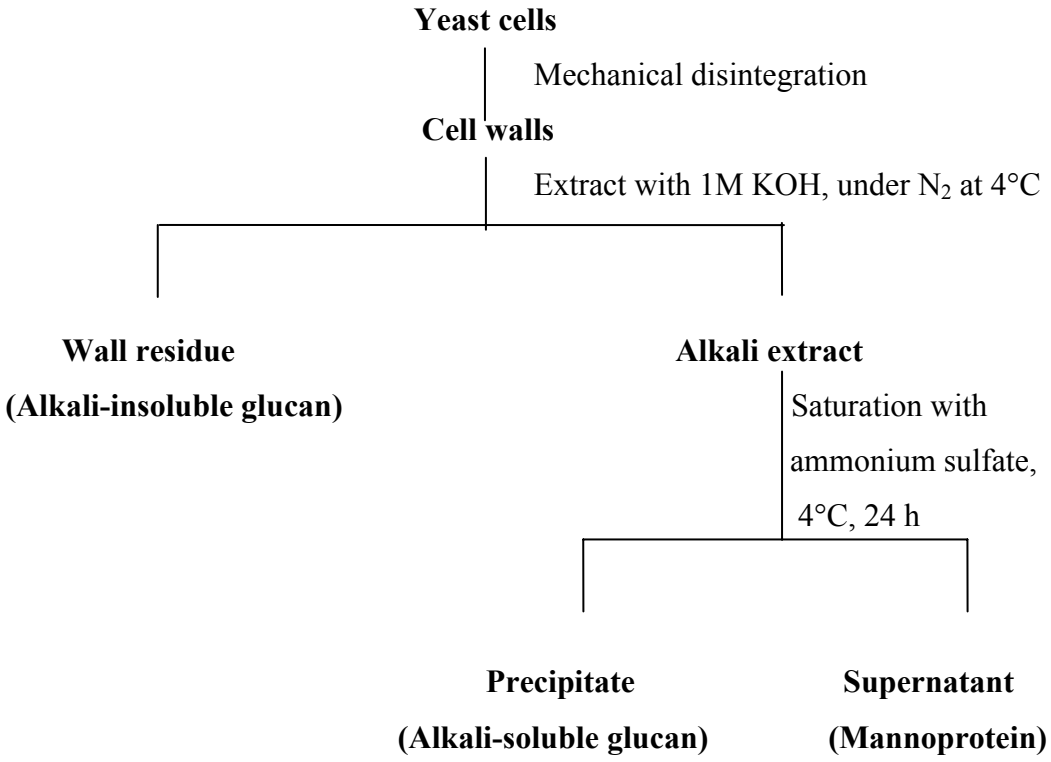


Figure 5. Schematic process of procedure for fractionation of yeast cell walls.
Source: Nguyen *et al*. (1998)

Suphantharika *et al.* (2003) extracted β-glucan from spent brewer's yeast. Yeast cells were autolyzed at 50°C for 24 h and the insoluble yeast cell wall fraction was obtained. It was used as a raw material for the preparation of β-glucan by alkaline extraction using 5 volumes of 1.0 N NaOH at 90 °C for 1 h.

Liu *et al.* (2008) studied a new mild method to extract β-D-glucans from *S. cerevisiae* cells. This method composed of induced autolysis, gentle extractions by water and organic solvents, homogenization and protease hydrolysis. The treatment procedure is illustrated in Figure 6. This process steps avoiding chain degradation and resulting in remaining of the native glucan structure.

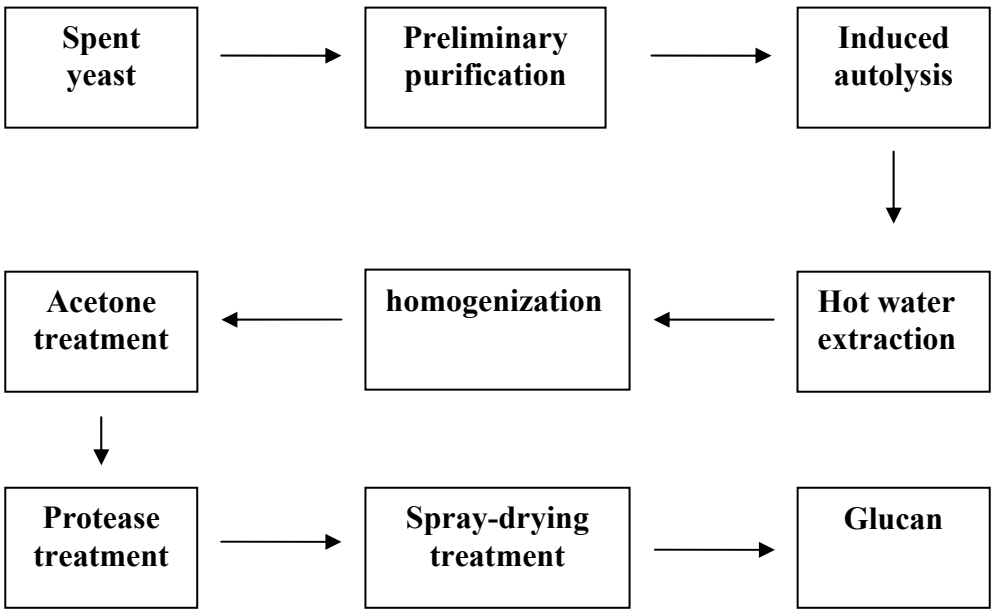


Figure 6. Scheme for preparation of β-D-glucans from spent yeasts.
Source: Liu *et al*. (2008)

6.2.3 Extraction by enzyme

An alternative to the drastic conditions mentioned is the treatment with enzymes. Glucanases, chitinases and proteases have been used to solubilize the components of yeast cell walls. Enzymatic treatments have the advantage of possibly preserving the native structure and avoiding chain degradation on the other hand, it is expensive (Freimund *et al*., 2003).

Mannoprotein was extracted from whole cells of bakers' yeast by digestion with Zymolase (a β-1,3 glucanase), incubated for 3 h at 25°C with gentle shaking. Insoluble material was then removed by centrifugation. Crude mannoprotein was precipitated from the supernatant with ethanol. It was found that crude emulsifier extracted from bakers' yeast by Zymolase contained approximately 38% carbohydrate and 16.6% protein. Treatment with Zymolase degrades the glucan component of the cell wall, and would therefore be expected to release the structural mannoprotein. (Cameron *et al*., 1998).

Alexandre *et al*. (2000) analyzed of velum-forming yeast cell wall component. Solubilized cell wall mannoprotein by β-1,3 glucanase treatment. The wet weight of cells were resuspended in 10 mM Tris-HCl pH7 extraction buffer containing

1 mM phenyl methyl sulfonide. The cells were then broken with glass beads (0.45 mm) using a Braun homogenizer. The lysate obtained was centrifuged and washed with the extraction buffer containing 10 mM dithiothreitol. The suspension was then placed under agitation during 45 min at 28°C. The cell walls pelleted was washed three times with extraction buffer and the pellet was resuspended in extraction buffer containing (400U/ml) β-1,3 glucanase after lyticase digested, the supernatant was recovered. After that partial purification of mannoprotein by anion exchange HPLC followed by surface hydrophobicity determination revealed that the fraction containing the 49 kDa mannoprotein which had the most hydrophobic.

6.3 Autolysis

Autolysis has been defined as the hydrolysis of intracellular biopolymers under the effect of hydrolytic enzymes. Hence, the temperature, pH and autolysis promoter should have profound effects on cell autolysis such as NaCl (Liu *et al*., 2008). Autolysis by endogenous enzyme occurs naturally in yeast when they complete the cell growth cycle and enter the death phase. In autolysis or self-digestion, the intracellular enzyme break down proteins, glycogen, nucleic acids, and other cell constituents. Mannoproteins can released into the medium, the yeast cell wall must be thoroughly degraded, and this is usually accomplished by an enzyme known as β-1,3 glucanase. This enzyme is produced by the yeast and has been shown to be present and active in the biomass and can be secreted into the surrounding media. However, it was released from dead cells after 8 weeks storage of the culture supernatant on yeast lees, may require months or years to occur. Following cell death, the process of autolysis is responsible for a gradual enzymatic cell wall degradation (Dupin *et al*., 2000; Ferreira *et al*., 2010). Charpentier and Feuillat (1993) have studied the autolysis process and describe it is occurring in the five stages. Stages 1 and 2 are directed toward cell membrane integrity and hydrolytic enzyme activation, and stages 3, 4 and 5 are concerned with the enzymatic degradation of intracellular macromolecules, the increase of cell wall porosity, and the degradation of compounds released into the medium.

7. Properties of Emulsifiers

Emulsions have been defined as heterogeneous systems of one liquid dispersed in another in the form of droplets (Becher, 1965). The two liquids are immiscible, one of which is dispersed as finite globules in the other. The dispersed phase is also called “internal phase” and the continuous phase is often refered to as “external phase”. When the two immiscible liquids are blended, a temporary emulsion may form. Both phases initially tend to form droplets. When the agitation is stopped, the droplets quickly coalescence, and the two liquids separate. Unless if a stable emulsion is to be produced, some third material must be present. Termed the emulsifying agent which, by its presence at the interface, prevents coalescence of the oil globules (Schramm, 2005). An emulsifier consists of water-soluble hydrophilic parts and water-insoluble lipophilic parts within its. When an emulsifier is added to a mixture of water and oil, the emulsifier is arranged on the interface, anchoring its hydrophilic part into water and its lipophilic part into oil. On the interface surface of water and air and of oil and air, the hydrophilic part and the lipophilic part are adsorbed and arranged around the interface. The interfacial tension is reduced by the emulsifier. That is, the force to separate the oil and water is thus weakened, resulting in the easily mixing of oil and water. Even though reduction of interfacial tension lowers the interfacial free energy produced on dispersion, it is the role of emulsifying agents as interfacial barriers that is most important (Lieberman *et al*., 1988).

7.1 Hydrophilic lipophilic balance (HLB)

The hydrophilicity and lipophilicity are different among emulsifiers, and the balance between the two is called “HLB value”. The value ranges from 0 to 20 (Larsson and Friberg, 1990). An emulsifier with higher lipophilicity shows a lower HLB whereas higher hydrophilicity has high HLB, and the behaviors and functions to water depend on this HLB. The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsifiers, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification. The HLB

value conception is an often used tool for the emulation choice (Lieberman *et al*., 1988).

All compounds that have hydrophilic parts and lipophilic parts are not always able use as an emulsifier (Table 6). The HLB value is calculated as follow (Oberbremer *et al*., 1990):

$$\text{HLB value} = 20 \times \frac{\text{(molecular weight of the hydrophilic part)}}{\text{(molecular weight of the whole molecule)}}$$

Table 6. Characteristic behaviors related to water of hydrophilic lipophilic balance (HLB) value.

Characteristic behaviors related to water	HLB	Ratio		Function	
		hydrophilic part	lipophilic part		
Not dispersing	0	0	100		
				Anti-foaming agent	
Slightly dispersing	2	10	90		
	4	20	80		W/O emulsification
	6	30	70		
Milky dispersion	8	40	60	Wetting agent	
Stable milky dispersion	10	50	50		O/W emulsification
Transparent dispersion	12	60	40		
				Cleaning agent	
Colloidal solution	14	70	30		
	16	80	20	Solubilizing agent	
	18	90	10		
	20	100	0		

Source: Lieberman *et al*. (1988)

7.2 Micelle

Because an emulsifier has opposite properties; hydrophilic and lipophilic, its solution does not become a simple aqueous solution but a colloidal solution, of which properties greatly vary depending on its concentration. In an extremely-diluted solution, there is no special change, but the emulsifier gathers on the interface and the surface tension is reduced as an increase of its concentration. As further increase of the concentration, a uniform mono molecular layer is made on the surface and the surface tension drops to the minimum. A further increase of the concentration causes micelle formation, micelles formation occurs when the excess molecules, in which the lipophilic groups are positioned face to face gather and there is no change in the surface tension.

The concentration to start micelle formation is called "critical micelle concentration (CMC)" and the properties of the solution change greatly with a change of this concentration (Fig.7). (Friberg and El-Nokaly, 1985).

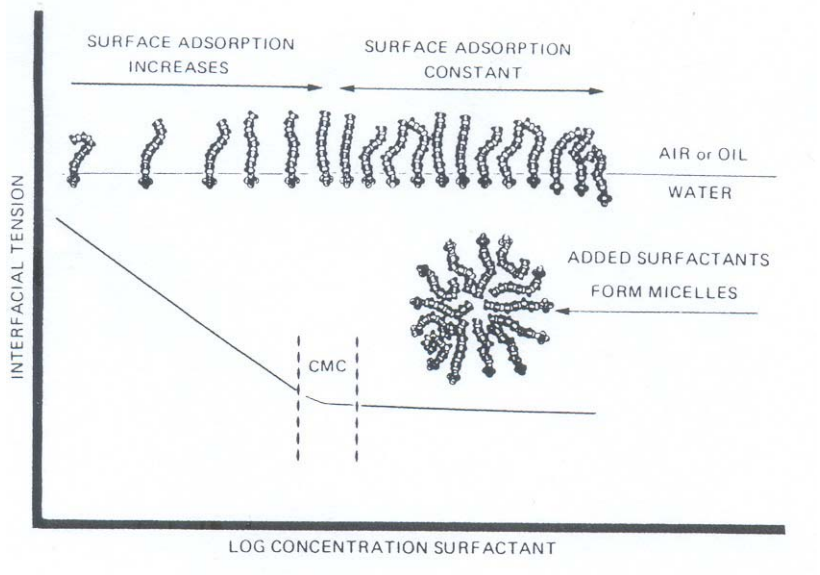


Figure 7. The critical micelle concentration (CMC).
Source: Friberg and El-Nokaly (1985)

7.3 Type of emulsion formed

Emulsion type is an important consideration in the preparation of emulsions, especially those intended for topical use. There are two common types of emulsion, depending on the amount of the two phases, the nature of the emulsifier, and the way it is prepared the nature of emulsifiers is described by a concept known as the hydrophile-lipophile balance (HLB), which is based on the relative fractions of water-compatible and oil-compatible groups in the surface-active agent (Rhein *et al.,* 2007).

1. Oil-in-water emulsion (oil droplets in water): water being the continuous phase surrounding droplets of oil such as ice cream, cream and milk drink. HLB value between 8-18.
2. Water-in-oil emulsion (water droplets in oil): micro-size droplets of water are completely surrounded by oil such as margarine, butter and butter cream. HLB value between 4-6.

Whether an emulsion turns into a water-in-oil emulsion or an oil-in-water emulsion depends on the volume fraction of both phase and on the type of emulsifier. Emulsifier tends to promote dispersion of the phase in which they do not dissolve very well. For example, if sample dissolve better in water than in oil, so tend to form oil-in-water emulsions. That is they promote the dispersion of oil droplets throughout a continuous phase of water (Lieberman *et al*., 1988).

8. Protein-Polysaccharide Interactions

Proteins and polysaccharides are the two kinds of biopolymers used by food technologists to control structure, texture and stability. Proteins are known for their emulsification and foaming properties, and the polysaccharides for their water-holding and thickening properties (Dickinson, 1998). A review of literature evidence suggested that much of the reported emulsifying capability of polysaccharides is explicable in terms of complexation or contamination with a small fraction of surface-active protein such as gum arabic that contains a small amount of protein. The more hydrophobic protein chain firmly anchors the protein-polysaccharide hybrid at the interface, and the protruding hydrophilic carbohydrate blocks attached to this chain provide a strong steric barrier towards flocculation and coalescence. Whilst charged

groups provide the basis for some electrostatic contribution to the colloidal stabilization (Dickinson, 2003).

For a polymer to be effective as an emulsifying agent, it must be surface-active. That is, it must have the capacity to lower the tension at the oil-water interface. Proteins have a strong tendency to adsorb at oil-water interface to form stabilizing layer around oil droplets. There are able to fulfill both the emulsifying and stabilizing role. Protein-polysaccharide interaction may enhance emulsion stability by forming a thicker and stronger steric stabilizing layer, or it may destabilize the emulsion by forming polymer bridge between flocculated droplets (Dickinson and McClements, 1996). Electrostatic complexation between protein and charge polysaccharide in emulsion systems allows associate adsorption of polysaccharide onto the surface of protein-coated oil droplets, leading to the potential for enhanced emulsion stabilization by the bulky secondary layer of charged polysaccharide molecule via conbined electrostatic and steric stabilization mechanisms (Semenova *et al.,* 2009).

There are many literature reviews evidence that much of the reported of polysaccharide-protein complex bioemulsifier from microorganisms:

Candida lipolytica produces extracellular water-soluble emulsifier "liposan". The purification procedure included repeated solvent extractions (chloroform-methanol-water) of a concentrated culture filtrate and Affi-Gel concanavalin; an affinity chromatography. Purified liposan composed of approximately 83% carbohydrate and 17% protein. The sugar content of liposan is similar to that of the emulsifier emulsan, which also contains glucose, galactosamine and a uronic acid in its carbohydrate moiety. The ability of liposan to emulsify and stabilize a number of commercial vegetable oils was examined. Liposan was most effective in stabilizing cotton seed oil, corn oil, soybean oil and peanut oil emulsions. Several commercial emulsifiers-stabilizers were examined for their emulsification and stabilization properties, using hexadecane as the water-immiscible oil. Of the thirteen agents examined, liposan ranked sixth in its effectiveness as an emulsifier and eighth as a stabilizer (Cirigliano *et al*., 1985).

Mannoprotein with emulsification properties was extracted from the cell walls of *K. marxianus* grown on a lactose-based medium by autoclaving cells in a

citrate buffer at pH 7. The composition of this mannoprotein was 90% carbohydrate (mannan) and 4-6% protein. These values are similar to mannoprotein extracted from cells of *S. cerevisiae*, emulsion were stable for 3 months in a range of pH (3-11) and NaCl concentrations (2-50 g/l) (Lukondeh *et al*., 2003).

Vasallo *et al.* (2006) characterized cell wall protein obtained from *Kluyveromyces fragilis* by alkali extraction, acid precipitation and chromatographic separation. Their surface and emulsifying properties can be explained on the bases of protein/carbohydrate ratio and water solubility. These results provide useful information for further applications at the food industry.

Paraszkiewicz *et al*. (2002) found that *Curvularia lunata* IM2901, the filamentous fungus used for hydrocortisone manufacture, has also an ability of bioemulsifier production with a yield of 2.6 g/l. These agents caused the formation of stable emulsions with hydrophobic components of natural and xenobiotic origin. Vegetable and mineral oils were the best substrates for emulsification. These strain released emulsifying agent from the exponential phase to the middle of the stationary phase. These bioemulsifier produced by these strain was a heteropolymer, which consisted of 48% polysaccharide and 25% protein parts but no fatty acids.

A novel bioemulsifier, Yansan, from *Yarrowia lipolytica* IMUFRJ 50682 has been successfully isolated from a glucose-base medium and has been evaluated for its emulsification properties. Yansan consists of a polysaccharide-protein complex with low lipid content with high emulsification activity and stability in a wide pH range (3-9). It has been demonstrated that the protein content of these polymers plays an important role in the emulsification activity. Moreover, Yansan was capable to form oil-in-water emulsions of aromatic hydrocarbons, such as toluene, xylene and stylene, with very high emulsification activities. Yansan was capable to form emulsions of both aromatic and aliphatic hydrocarbons. This surfactant shows potential application in a variety of fields, especially in bioremediation and formulation of perfluorocarbon-based emulsions (Amaral *et al*., 2006).

9. Utilization of Bioemulsifier

Waters *et al*. (1994) isolated a *S. cerevisiae* yeast mannoprotein from red wine. It was purified by combination of anion exchange, concanavalin A, cation exchange and gel permeation chromatography. It apparent molecular weight when compared with pullulan standard was 420 kDa. It can protect wine from protein haze spoilage. The discovery of this yeast mannoprotein with haze protective property can be commercially exploited.

Shepherd *et al*. (1995) tested a range of microorganisms for production of extracellular bioemulsifier. Of the total of 24 microbes, seven showed no emulsification activity, nine organisms produced extracellular emulsifiers that were as good as the positive controls gum arabic and carboxymethylcellulose and eight organisms produced extracellular emulsifiers that were better than gum arabic and carboxymethylcellulose. The eight good producer organisms included the yeasts *Candida utilis, Candida valida, Hansenula anomala, Rhodospiridium diobovatum,* and *Rhodotorula graminis,* the red alga *Porphiridium cruentum,* and the bacteria *Klebsiella* spp. and *Acinetobacter calcoaceticus.* Crude preparations of the bioemulsifier from *C. utilis* exhibited low viscosity and had a carbohydrate content of over 80%. Preliminary trials showed that the bioemulsifier from this organism had potential for use in salad cream. Salad creams prepared using 0.2% and 0.8% w/v of the bioemulsifier were shown to separate to a lesser extent than controls prepared with reduced amounts of egg and no stabilizer (guar and xanthan gums) when stored at 4°C for 1 week.

Torabizadeh *et al*. (1996) extracted mannoprotein from the cell wall of *S. cerevisiae*. The mannoprotein consisted of approximately 380-410 g/kg protein and 210 g/kg carbohydrate. Emulsification tests on mannoprotein revealed that its function is similar to the sodium caseinate. Mannoprotein and sodium caseinate showed the emulsion of 79% and 77%, respectively. In addition, it has remain emulsion phase after 1 month at 4°C 78% and 75%, respectively. Practical application of this bioemulsifier for mayonnaise production with several different formulations indicated that it can be used with carboxymethyl cellulose (CMC) (instead of xanthan) for production of mayonnaise.

Thammakiti *et al.* (2004) found that the β-glucan obtained from spent brewer's yeast had emulsion stabilizing capacity. This finding suggested that it can be used in food products as a thickening, water holding or oil-binding agent and emulsifying stabilizer. Consequently, Worrasinchai *et al.* (2005) applied to use β-glucan as a fat replacer in mayonnaise. Fat was partially substituted by β-glucan. The substitution levels of not more than 50% of oil used were found to be acceptable.

In addition, emulsifier can use in microencapsulation technique that is a process whereby particles of sensitive or bioactive materials are covered with a thin film of a coating material. The encapsulated substance (e.g. fats, oils, aromas, flavors) is usually refered to as the "core" material, whereas the film surrounding the core is usually called the "wall" material (Sheu and Rosenberg, 1995). The two major processing steps in microencapsulation of liquid flavorants are (1) Emulsification of the encapsulated substance into an aqueous dispersion of the microencapsulant that functions as an emulsifier. Hydrophobic core material is usually homogenized in the presence of an aqueous solution containing an emulsifier (e.g. surfactant, phospholipids or biopolymer) that forms a protective coating around the oil droplets, and then wall materials are mixed with the resulting emulsion. (2) Drying the microencapsulated emulsion under conditions that minimize loss of the encapsulated material by volatilization and that enhance the chemical stability of the encapsulated materials (Kim *et al.,* 1996).

Of many methods available, spray-drying largely dominates the market for encapsulation of flavours (Goubet *et al.,* 1998). Shaikh *et al*. (2005) reported on microencapsulation of black pepper oleoresin by spray-drying, using gum arabic and modified starch as wall materials. Due to sensitivity to light, heat and oxygen of spice oleoresins. Moreover, it has short storage lives if not stored properly. Microencapsulation protects the oleoresin against such destructive change. Besides, it also protects the flavours from undesirable interaction with food and minimize flavour/flavour interaction. Klinkesorn *et al*. (2005) prepared tuna oil-in-water emulsion containing droplets stabilized either by lecithin membranes (primary emulsions) or by lecithin-chitosan membranes (secondary emulsions). A primary emulsion containing small anionic droplets coats with a lecithin membrane is produced by homogenized oil and water together in the presence of lecithin, a low

molecular weight emulsifier that rapidly adsorbs to the surface of oil droplets during homogenization.

A secondary emulsion containing cationic droplets coated with a lecithin–chitosan membrane is then produced by adding chitosan to the primary emulsion. The production of emulsions containing droplets surrounded by multiple-layered interfacial membranes may prove to be an effective means of improving the stability of microencapsulated oils. Therefore, the interfacial engineering technology used in this study could lead to the creation of food emulsions with improved stability to environmental stress. Moreover, Papagianni and Anastasiadou (2009) developed encapsulation techniques of *Pediococcus acidilactici* cells in food grade encapsulation systems as carriers that ensure delivery of large populations of viable cells to a target point. The microorganisms were encapsulated in microscopic oil droplets emulsified with peptides and stabilized with xanthan in oil-in-water emulsions.

10. Microencapsulation Technology

Microencapsulation is defined as a technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under the influences of specific conditions. A microcapsule consists of a semipermeable, spherical, thin, and strong membrane surrounding a solid/liquid core, with a diameter varying from a few microns to 1 mm. In a broad sense, encapsulation can be used for many applications in the food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavours, colours or odours, extending the shelf life and protecting components against nutritional loss. Food-grade polymers such as alginate, chitosan, carboxymethyl cellulose (CMC), carrageenan, gelatin and pectin are mainly applied, using various microencapsulation technologies (Anal and Singh, 2007).

10.1 The encapsulation techniques

It can be classified into 2 groups, depending on the method used to form the beads: extrusion (droplet method) and emulsion or two-phase system.

10.1.1 Extrusion technique

Extrusion is the oldest and most common approach to making capsules with hydrocolloids. It simply involves preparing a hydrocolloid solution, adding microorganisms to it, and extruding the cell suspension through a syringe needle in the form of droplets to free-fall into a hardening solution or setting bath (Fig. 7). The size and shape of the beads depend on the diameter of the needle and the distance of free-fall, respectively. This method is the most popular due to its easy, simplicity, low cost, and gentle formulation conditions ensuring high retention of the cell viability (Krasaekoopt *et al.,* 2003).

The supporting material used for extrusion is alginate, which is a linear heteropolysaccharide of D-mannuronic acid and L-guluronic acid extracted from various species of algae. Depending on the source, the composition and the sequence in L-guluronic acid and D-mannuronic acid vary widely. The functional properties of alginate as supporting material correlate strongly with the composition and sequence of L-guluronic acid and D-mannuronic acid. Divalent cation such as Ca^{2+}bind preferentially to the polymer of L-guluronic acid. The length of the polymer of D-mannuronic acid is, therefore, the main structural feature contributing to gel formation.

To form beads, a cell suspension is mixed with a sodium alginate solution, and the mixture dripped into a solution containing a multivalent cation (usually Ca^{2+} in the form of $CaCl_2$). The droplets form gel spheres instantaneously, entrapping the cells in a three-dimensional lattice of ionically cross-linked alginate, the success of the alginate gel encapsulation technique is due to the gentle environment it provides for the entrapped material, cheapness, simplicity, and its biocompatibility.

The size of the beads is approximately 2-3 mm in diameter. Moreover, the size and sphericity of the bead depend mainly on the viscosity of the sodium alginate solution and the distance between the syringe and the calcium chloride collecting solution. As the concentration, and hence viscosity, of sodium alginate increases, the size of the beads decreases (Krasaekoopt *et al*., 2003).

10.1.2 Emulsion technique

In this technique, a small volume of the cell-polymer suspension (discontinuous phase) is added to a large volume of a vegetable oil (continuous phase) such as soybean oil, sunflower oil, canola oil or corn oil. The mixture is homogenized to form a water-in-oil emulsion. Once the water-in-oil emulsion is formed, the water-soluble polymer must be insolubilized (cross-linked) to form tiny gel particles within the oil phase (Fig. 8). The smaller the internal phase particle size of the emulsion, the smaller the final microparticles will be. The insolubilization method of choice depend on the type of supporting material used. The beads are harvested later by filtration. The size of the beads is controlled by the speed of agitation, and can vary between 25 μm and 2 mm. This technique has been used successfully to encapsulate lactic acid bacteria for batch and continuous fermentation.

- continuous phase

For food applications, vegetable oils are used as the continuous phase. Some studies have used white light paraffin oil and mineral oil. In some cases emulsifiers are added to form a better emulsion, because the emulsifier lower the surface tension, resulting in smaller spheres.

- supporting material

There are many supporting materials used with the emulsion technique. These include a mixture of K-carrageenan and locust bean gum, alginate, chitosan and gelatin.

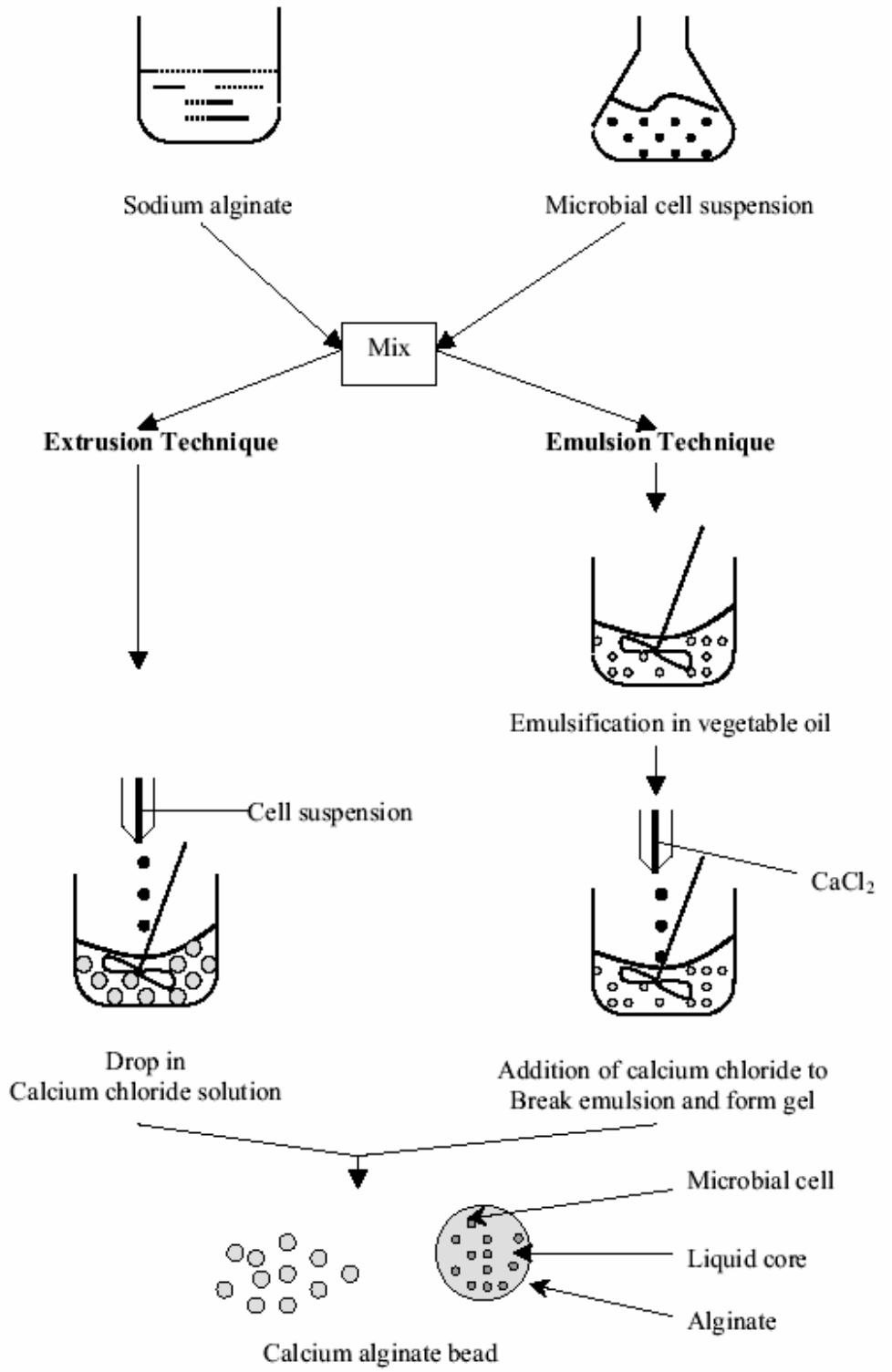


Figure 8. Flow diagram of encapsulation of bacteria by the extrusion and emulsion technique.

Source: Krasaekoopt *et al*. (2003)

Advantages and disadvantages of these techniques are shown in Table 7. Extrusion is a relatively simple technique. It usually produces entrapped, rather than encapsulated core material, although encapsulation can be achieved through co-extrusion deviced or dropping into a bath of coating material which react at the droplet surface. The method can be difficult for large-scale production because of slow formation of beads compared with the emulsion technique.

On the other hand, the emulsion technique is relatively new to the food industry and easy to scale up for large-scale production. It provided both encapsulated and entrapped core materials. The size of the beads formed by this method is smaller (25 μm to 2 mm) than that of beads produced by the extrusion method depends mainly on the size of the needle used, while the size of beads from the emulsion method depends on the speed of agitation and the type of emulsifier used. Due to the need for a vegetable oil, the operating cost of the emulsion technique may be higher than that of the extrusion technique.

Table 7. Positive and negative features of extrusion and emulsion techniques.

	Extrusion	Emulsion
Technological feasibility	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of microorganism	80-95%	80-95%
Size of bead	2-5 mm	25 μm-2 mm

Source: Krasaekoopt *et al*. (2003)

10.2 Microencapsulation with probiotic

Probiotics have been defined as “live microbial feed supplements that have beneficial effects on the host by improving their intestinal microbial balance” (Fuller, 1992). In order to be effective and confer health benefits to the host, probiotics must be able to survive passage through the stomach and upper intestine and be present in sufficient amount to impact the colon microenvironment. This means that they must tolerate the acidic and protease rich conditions of the stomach and survive and grow in the presence of bile acids. Therefore, to be effective and confer these

health benefits, probiotic cultures must be able to withstand processing conditions, retain their probiotic properties after processing, and survive in sufficient numbers in the product during shelf life/storage (Piano *et al*., 2006).

Over the last 20 years there has been an increased interest in the role of probiotic bacteria in human health. This has led to industries focusing on incorporating these lactic acid bacteria in dairy foods. Probiotics have been incorporated into a range of dairy products, including yoghurt, cheeses, ice cream, milk powders and frozen dairy desserts. However, there are still several problems with respect to the low viability of probiotic bacteria in dairy foods. The standard for any food sold with health claims from the addition of probiotic is that it must contain per gram at least 10^6 – 10^7 CFU of viable probiotic bacteria (FAO/WHO 2001). However, this lactic acid bacteria may not survive in sufficient numbers after pass through the gastric-intestinal tract (Hamilton-Miller, 1999). A major barrier to the survival of ingested microorganisms is the acidic envioronment (pH 2.0) of the stomach. The viable microbial content and general quality of many probiotic-containing products have often been questionable.

Analysis of products in several different countries has confirmed that probiotic strains exhibit poor survival in traditional probiotic foods such as yoghurt and fermented milks (Shah, 2000). Microbial behaviour (growth, survival and death) in foods is largely governed by properties of the food (water availability, pH and buffering capacity) in addition to the storage conditions (temperature, relative humidity and atmosphere) (McMeekin *et al*., 1997). Hence, technologies that can protect the viability of probiotics during manufacture, storage and gastrointestinal transit are highly desired. Microencapsulation of bacterial cells is currently gaining attention to increase viability of probiotic bacteria in acidic products. Microencapsulation technologies have been developed and successfully applied using various matrices to protect the bacterial cells from damage caused by the external environment guarantee their improved survival during gastro-duodenal transit, and enhance their stability profile (Piano *et al*., 2006).

10.3 Optimization of encapsulation parameters

10.3.1 Concentration of alginate

Chandramouli *et al.* (2004) developed microencapsulation method to increase the efficacy of capsules in protecting the encapsulated bacteria under simulated gastric conditions. They also found that the viability of the cells *Lactobacillus* spp. in the microcapsules increased with an increase gel concentration from 0.75% to 1.8% (w/v). There was no significant increase ($p>0.05$) in viable cell numbers of capsules when the alginate gel concentration was further increased to 2% (w/v). Alginate concentration beyond 2% (w/v) made uniform spherical capsule formation difficult because of increased viscosity. A higher concentrations of sodium alginate, better protection of bacterial cells was achieved. Increasing the biopolymer (sodium alginate) concentration increases the number of binding site for Ca^{2+} ions. As a results, a more densely cross-linked gel structure will be formed.

Four concentrations of calcium alginate were used to encapsulated *Lactobacillus bulgaricus* L2 cells. These alginate beads were suspended in distilled water and still frozen for 24 h. Concentration of alginate had a significant ($p<0.001$) effect on cell viability. Cells in beads of 6% calcium alginate had almost 100% survival, but only 50% of cells in 0.5% calcium alginate survived. Beads of 1.5 and 3% calcium alginate provided about the same ($p>0.05$) protection. Although cells survived best in beads of 6% calcium alginate, a solution with that high concentration was too viscous to handled under production (Sheu *et al*., 1993).

Lee and Heo (2000) entrapped *Bifidobacterium longum* KCTC 3128 and HLC 3742 in various sodium alginate concentration (2, 3 and 4% w/v). When the bifidobacteria entrapped in calcium alginate beads were exposed to stimulated gastric juices and a bile salt solution, the death rate of the cells in the beads decreased proportionally with an increase alginate gel concentration. It was found that gel concentrations below 2% did not form spherically shaped beads against drag forces upon collision with a solution. Whereas a high concentration of sodium alginate (5% or more) can not form small droplets because of its physiological characteristics as a dough. Accordingly, cell entrapment is limited to the range of gel concentrations that form spherical beads.

Similarly, the death rate of *Lactobacillus casei* NCDC-298, immobilized in alginate decreased proportionately with increasing alginate concentrations (2-4%). The highest survival of cell was recorded in 4% alginate beads, followed by 3% and 2%. Thus, viability of encapsulated *L.casei* NCDC-298 cells improved with increasing alginate concentration (Mandal *et al*., 2006).

The formation kinetics of calcium alginate gel capsules was studied by Blandino *et al.* (1999). An increase in the concentration of alginate was studied by fixing the cationic solution gives rise to a reduction in membrane thickness, while an increase in the concentration of calcium chloride leads to the formation of a thicker film. This effect is presumably due to the fact that on increasing the number of biopolymer molecules per unit solution volume in the vicinity of the core capsule, the number of binding site for Ca^{2+} ions also increases. As a result, a more densely cross-linked gel structure will probably form and consequently, it will have a smaller thickness.

10.3.2 Concentration of $CaCl_2$

The effect of $CaCl_2$ concentration on capsule formation kinetics was studied by fixing the anionic solution. On increasing calcium chloride concentration, the thickness of the membrane increases. This result can be explained by the fact that an increase in the mass of calcium ions initially contained in the core capsule will result in a larger concentration gradient between the core and the outside solution. This situation will favour the diffusion of Ca^{2+} ions from the core. Moreover, the time required to obtained the maximum gel film thickness is considerably longer when calcium chloride concentration exceeds 5.5% w/v. This result confirms that the membrane thickness increases continuously until complete consumption of calcium ions contained in the core capsule has been achieved (Blandino *et al.,* 1999).

Chandramouli *et al.* (2004) indicated that increasing the calcium chloride concentration is not important in increasing the viability of the encapsulated cell under simulated gastric condition. Tanaka *et al*. (1984) reported that concentration of calcium chloride has little effect on the diffusion characteristics of the beads. Drop wise addition of anionic solution into the calcium chloride solution results in an instantaneous capsular membrane. However, when diffusing through a gel that has

already formed where all the binding sites are occupied, there is no opportunity for Ca^{2+} ions to bind until they reach additional available binding sites in the gelling zone.

10.3.3 Capsule size

Results from the studied of Chandramouli *et al.* (2004) indicated that viability of encapsulated bacteria *Lactobacillus* spp. in simulated gastric conditions increased with increased capsule size (200-1000 μm). Similar improvements in survival have been reported for *lactobacilli* encapsulated in alginate microspheres by other workers. In relation to bead size, the survival of cells in beads is higher with larger beads, large, medium, and small beads (mean diameters about 1.03, 1.75 and 2.62 mm, respectively). Sheu *et al.* (1993) indicated that larger bead diameters provided more protection for *Lactobacillus bulgaricus* in frozen desserts. Very large beads, however, can cause a coarseness of texture in live microbial feed supplements, and small beads cannot provide sufficient protection for the bacteria. Also, Hansen *et al.* (2002) reported that very large beads (1000 μm) cause a coarseness of texture in live microbial feed supplements and small beads of size less than 100 μm is able protect probiotics in food products but not in the more than extreme environment found in low pH simulated gastric juice. Moreover, Lee and Heo (2000) found that the death rate of entrapped *Bifidobacterium longum* in the beads decreased proportionally with increased bead size.

10.4.4 Cell loading

Increasing in cell load during encapsulation increased the number of bacterial survivors at the end of 3 h incubation in simulated gastric conditions. (Chandramouli *et al*., 2004). Whereas, Lee and Heo (2000) found that the initial cell numbers did not affect the death rates of the viable cells.

10.4.5 Hardening the capsule in calcium solution

Results from the studied of Chandramouli *et al.* (2004) showed that there was a significant decrease in viable cells of encapsulated *Lactobacillus* spp. (after 3 h incubation at pH 2), which had been hardened in 0.1 M calcium chloride solution for 5 min compared to those that has been hardened for 30 min or more.

Hence, it is recommended that the capsules should be hardened in 0.1 M calcium chloride solution for at least 30 min before use, increasing encapsulated cells' stability.

It was found that the thickness of membrane increases rapidly within the first 15 min of the process. Indeed, within the first 10 min, membrane thickeness is about 50% of its maximum value. After this first stage, the thickness of the gel film increases more slowly, and finally levels off at its maximum value. This kind of behaviour indicates that gel film formation occurs from outside the core of the capsule. All these results can be easily explained by taking into consideration the gel formation process, which is assumed to be controlled by the diffusion of the two components involved in it. In this regard, the fact that the cation has a smaller size than the polymer molecules means that it is mainly the cation that diffusion between the alginate chains binding to unoccupied binding sites on the polymer. Thus, once the cationic solution is added dropwise into the alginate solution, a capsular membrane forms instantaneously around the droplet, and this will grow along the flux direction of the Ca^{2+} ions. When the mass of Ca^{2+} ions contained within the core capsule is exhausted, the gelation process is finished. As far as the initial moments of the gel formation process are concerned, all the binding sites for Ca^{2+} ions present in alginate chains are unoccupied, so cations can bind rapidly to the polymer. However, when diffusing through a gel that has already formed where all the binding sites are occupied, there is no opportunity for Ca^{2+} to bind until it reached available binding site in the gelling zone. Thus, calcium ions must diffuse through the gel film to reach with sodium alginate during the formation of capsules. This means that the maximum growth of the gel film is realized within the first 15 min of the process, where the resistance to diffusion caused by the gel film is not significant (Blandino *et al*., 1999).

Objective of study

1. To isolate yeast from palm wine fermentation and identify the isolated yeast base on 26S rDNA sequence.
2. To extract and characterize the bioemulsifier from spent yeast obtained from traditional liquor distillation.
3. To purify the bioemulsifier from spent yeast obtained from traditional liquor distillation.
4. To study the composition of the bioemulsifier from spent yeast obtained from traditional liquor distillation.
5. To use the bioemulsifier to encapsulate probiotic bacteria isolated from *Kung-Som* by microencapsulation technique.

CHAPTER 2

BIOEMULSIFIER FROM SPENT YEAST OBTAINED FROM THAI TRADITIONAL LIQUOR DISTILLATION: EXTRACTION AND CHARACTERIZATION

2.1 Abstract

Bioemulsifier yield of 0.53 g/g dry cells was obtained from spent yeast, which was a by-product from Thai traditional liquor distillation by autoclaving in a neutral citrate buffer pH of 7.0 at 121°C under pressure of 15 psi for 30 min. The spent yeast bioemulsifier obtained was evaluated for chemical and physical stability to establish its potential use as a natural emulsifier in processed foods. The extracted spent yeast bioemulsifier exhibited emulsion activity of 60.23% towards palm oil as oil-in-water and had a critical emulsifier concentration of 20 g/l. The composition of the spent yeast bioemulsifier was 96% carbohydrate and 4% protein. The emulsion activity of the spent yeast bioemulsifier was compared to those of commercial emulsifiers (lecithin and gum arabic). The spent yeast bioemulsifier can emulsified more varieties of vegetable oil than lecithin and gum arabic. The emulsion activity of the spent yeast bioemulsifier towards palm oil was stable over a broad range of pH (3-12), NaCl concentrations of 0-3% (w/v), $CaCl_2$ and $MgCl_2$ concentrations of 0-0.1% (w/v). Temperatures (4°C, 10°C, room temperature, 63°C, 100°C, and 121°C) did not affect the emulsion activity of the spent yeast bioemulsifier. The bioemulsifier from spent yeast shows good potential for using in the food industry.

2.2 Introduction

Emulsifiers are predominantly employed by the food manufacturers to stabilize a mixture of immissible substances. In addition to its function importance, emulsifiers offer various other advantages to the food manufacturers, which include: extended shelf life for processed foods, improved organoleptic properties (texture, flavor, and mouth-feel) and increased production flexibility and efficiency. Food emulsifiers can be classified as two broad classes: natural and synthetic emulsifiers. A large number of emulsifiers are natural products derived from plant or animal sources. Lecithin, the most widely marketed natural emulsifier, is derived from vegetable oils. Synthetic emusifiers are derived from chemical reactions between fatty acids and glycerol. However, foods made by using such synthetic emulsifier have an unpleasant flavor and have a taste of emulsifier used, which deteriorates the original flavor and taste of the food themselves. In addition, synthetic emulsifiers are suspected to cause health problems which may be derived from the continuous intake of the foods containing such substances for a long period of time. Thus, it becomes of urgent necessity to replace these various synthetic substances for foods with naturally substances (Hasenhuettl and Hartel, 2008). Thus natural emulsifiers are becoming increasingly important in the food industry rather than the synthetic ones which may be potential health hazards for humans (Lukondeh *et al*., 2003).

Currently Thai government promotes “One Tumbol-One Product (OTOP) policy” and one of OTOP products is a traditional distilled spirit. Generally, producers directly distill palm sugar wine without separation of yeast cells to obtain liquor. After distillation, a huge amount of waste containing yeast cells is discharged. This causes environmental problems because it has a high biological oxygen demand. *Saccharomyces cerevisiae* is normally used for alcohol fermentation. Mannoprotein extracted from *S. cerevisiae* has been shown to be an effective bioemulsifier (Cameron *et al*., 1988). The presence of hydrophilic mannan polymers covalently attached to the protein backbone provides the mannoprotein with the amphiphilic structure common to surface active agents and effective emulsifiers (Cooper and Goldenberg, 1987). Mannoprotein is an emulsifier obtained as a by-product from the wine or brewing industry. It is readily availability, biodegradable, is not toxic, and large scale

production is possible. It can make possible the producing of value-added by-products (Torabisadeh *et al*., 1996). Since *S. cerevisiae* is edible and used in the manufacture of food and beverage products, it is assumed that a mannoprotein bioemulsifier would be non-toxic and generally recognized as safe (GRAS) (Cameron *et al*., 1988).

The objectives of this study were to extract and characterize bioemulsifier from spent yeasts obtained from Thai traditional liquor distillation. The emulsifier property of the spent yeast bioemulsifier was also compared with those of commercial bioemulsifiers, lecithin and gum arabic.

2.3 Materials and Methods

2.3.1 Chemicals

Commercial vegetable oils (soybean oil, palm oil, corn oil, olive oil, sunflower oil, rice bran oil and sesame oil) were purchased from a supermarket. Gum arabic was purchased from Nacalai Tesgue, Inc. (Kyoto, Japan). Lecithin was obtained from Fluka (USA). All other chemicals used were analytical grade.

2.3.2 Extraction and partial purification of the spent yeast bioemulsifier

Distillate bottoms obtained from local distillery in Songkhla Province, Thailand. It was centrifuged at 6,000×*g* for 10 min at 4ºC. Cells pellet were washed twice in normal saline. Twenty percent (w/v) yeast cells were suspended in distilled water containing 0.1 M potassium citrate and 0.02 M potassium metabisulfite. The pH of the suspension was adjusted to 7 with 1 M NaOH. The spent yeast bioemulsifier was extracted by two different heat conditions. First, the cell suspension was extracted by actoclaving at 121°C under pressure of 15 psi for 15, 30, 60, 90 and 120 min. Second, the cell suspension was extracted by only heating at 125°C for 3, 4 and 5 h. The resulting suspensions of two conditions were centrifuged at 6,000×*g* for 10 min at 4ºC. The supernatant was retained and mixed with five volume of chilled ethanol, incubated overnight at 4ºC for complete precipitation. The suspension was centrifuged at 6,000×*g* for 10 min at 4ºC. After centrifugation, the supernatant was discarded and the precipitate was washed twice with chilled ethanol. The precipitate was dried by a rotary evaporator and freeze dried (Barriga *et al*., 1999).

2.3.3 Determination of type of emulsion formed

Filter paper wetting test and dilution test (Rieger, 1986) were used to determine the type of emulsion formed. For the filter paper wetting test, droplet of emulsion was dropped onto filter paper. If the emulsion is water-in-oil (w/o), droplet of emulsion would remain a droplet on the filter paper. If the emulsion is oil- in-water (o/w) droplet of emulsion would disperse rapidly on the filter paper. For the dilution test, droplet of emulsion was dropped into water and oil. If the emulsion is w/o, droplet of emulsion would disperse in the oil and remain a droplet in the water. If the emulsion is o/w, droplet of emulsion would disperse cloudy in the water and remain a droplet in the oil.

2.3.4 Determination of droplet size distribution

The droplet size distributions (DSD) for fresh emulsions were measured approximately 1 h after the preparation by a laser diffraction method as described by Hayati *et al*. (2007). Distilled water was used as the dispersant for the determination of the emulsion lipid globule size distribution. The software used a reflective index of dispersant RI 1.33 (water) to calculate the Dispersion Index (Span) by span = d(90)-d(10)/d(50). The d(10), d(50) and d(90) values are size values corresponding to the cumulative distribution at 10%, 50% and 90%, respectively. Thus, the d(10) represents a size value below which 10% of the cumulative distribution is present. Drops of emulsion were introduced into the sample presentation unit until the concentration reached the optimum one, as indicated by the instrument.

2.3.5 Stability of the spent yeast bioemulsifier

The spent yeast bioemulsifier (20 g/l) was prepared in distilled water. To investigate the effects of pH, salts concentration (NaCl, $CaCl_2$ and $MgCl_2$) and temperature on emulsion activity of the spent yeast bioemulsifier, the spent yeast bioemulsifier solution was adjusted with 1 N HCl or NaOH to obtain the pHs of 3-12. NaCl was added to the sample to obtain the final concentrations of 0-3% (w/v). $CaCl_2$ and $MgCl_2$ were also added to the samples to obtain the final concentrations of 0-0.1% (w/v). For thermal stability study, the spent yeast bioemulsifier solution was incubated at 63ºC for 30 min, 100ºC for 15 min and 121ºC for 15 min and cooled to 30ºC. The remaining activity of the spent yeast bioemulsifier was then determined. Moreover, the

effect of temperatures on stability of the emulsion between room temperature, 4°C and 10°C were studied. Commercial bioemulsifiers (lecithin and gum arabic) at the same concentrations were also subjected to stability study.

2.3.6 Analytical methods

The total carbohydrate was determined colorimetrically by the method of Dubois *et al*. (1956) with mannose and glucose used as the standard. Protein was estimated by the dye-binding assay (Bradford, 1976) with bovine serum albumin used as the standard. Emulsion activity (emulsification activity and emulsification index) was measured according to the method of Cameron *et al.* (1988) with a slight modification. To 1 ml of the spent yeast bioemulsifier suspension, 1 ml of vegetable oil was added and vortexed at high speed for 3 min. The mixture was allowed to stand for 1 h (emulsification activity, %EA) and 24 h (emulsification index, $\%E_{24}$) prior to measurement. Emulsion activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage. The experiments were done in triplicate and results were reported as the average from triplicate determinations.

2.3.7 Statistical analysis

Data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan's multiple-range test. Statistical analysis was performed using the Statistical Package for Science (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL).

2.4 Results and Discussion

2.4.1 Extraction and partial purification of the spent yeast bioemulsifier

The effect of the extraction time by different conditions on the bioemulsifier yield, emulsification index ($\%E_{24}$) and critical emulsifier concentration of the spent yeast bioemulsifier are summarized in Table 8. It was found that the yield of the spent yeast bioemulsifier increased with increasing the time in the autoclaving at 121°C under pressure of 15 psi. However, every extraction time had E_{24} at about 60%. Critical emulsifier concentrations were 20 g/l and 30 g/l when autoclaved for 15-60 min and 90-120 min, respectively. The effectiveness of the bioemulsifier is evident from its low critical micelle concentration (Mohana *et al*., 2009). Although

autoclaving for 15 min also gave the least critical emulsifier concentration and exhibited the same emulsification activity value, it gave less stability of emulsion after being left to stand at room temperature for many days (data not shown). Accordingly, autoclaving for 30 min was the best condition for the bioemulsifier extraction from spent yeast obtained from traditional liquor distillation. In the present study extraction time was shorter than that of Torabizadeh *et al*. (1996) who found that the optimum time for *S. cerevisiae* bioemulsifier extraction was 120 min at 121ºC. This might be due to the effect of heating during the liquor distillation process. The distillers boil the palm wine for 2 to 3 h to obtain distilled spirit. Therefore, for spent yeast obtained from the waste of traditional liquor distillation it was not necessary to spend as a long time for extraction as with fresh yeast cells. Besides, bioemulsifier are freely soluble in water (Barriga *et al*., 1999) and can be extracted by using citrate buffer that contained 0.1 M potassium citrate and 0.02 M potassium metabisulfite. A reducing agent, potassium metabisulfite, was included in the heat extraction buffer as it increased the emulsifier yield. It can prevent from oxidation reaction of substance. Heat extraction procedure at neutral condition by adjusted the pH of the mixture to 7 is expected to solubilized the structural bioemulsifier in the outer layer of the cell wall (Cameron *et al*., 1988). Moreover, the extraction by autoclaving that included heat and pressure in the process easier to solubilized bioemulsifier than the extraction method that used only heat (125°C) (Table 8). It got higher yield and emulsion activity. As a result, this extraction procedure (actoclaving at 121°C) is simple and suitable for large-scale production. It was found that the bioemulsifier obtained from spent yeast got yield 0.53 g/g of dry cells and consisted of a minor proportion of 4% proteins which was covalently linked to the major proportion of 96% carbohydrates. The carbohydrate and protein content in this study was consistent with the report of Lukondeh *et al.* (2003) who found that mannoprotein was composed of 90% carbohydrate (mannose) and 4-6 % protein.

Table 8. Effect of extraction time by different heating conditions on the spent yeast bioemulsifer yield, emulsification index (%E_{24}) and critical emulsifier concentration.

Extraction time (min)	Spent yeast bioemulsifier yield* (g/g dry cell weight)	Emulsification index (%E_{24})	Critical emulsifier concentration (g/l)
Heating at 121°C under pressure of 15 psi			
15	$0.523 \pm 0.004^{B**}$	60.71 ± 3.57^{A}	20
30	0.534 ± 0.009^{B}	60.23 ± 1.74^{A}	20
60	0.537 ± 0.003^{B}	60.78 ± 1.32^{A}	20
90	0.568 ± 0.021^{A}	60.50 ± 1.15^{A}	30
120 (2 h)	0.577 ± 0.012^{A}	60.27 ± 2.93^{A}	30
Heating at 125°C			
3	$0.499 \pm 0.015^{a**}$	51.23 ± 2.97^{a}	20
4	0.504 ± 0.016^{a}	51.19 ± 4.37^{a}	30
5	0.520 ± 0.018^{a}	54.99 ± 0.99^{a}	30

* Values are given as mean ± SD from triplicate determinations.

** Different letter in the same column indicate significant differences ($p<0.05$).

2.4.2 Substrate specificity of the crude spent yeast bioemulsifier compared with commercial emulsifier

The spent yeast bioemulsifier, gum arabic and lecithin at the critical emulsifier concentration (20 g/l) was checked for the specificity of vegetable oil emulsion. The spent yeast bioemulsifier emulsified all vegetable oils tested (Table 9). The maximum emulsifying activity was observed with olive oil, corn oil, sunflower oil and palm oil. However, the emulsification index of the crude spent yeast bioemulsifier towards these vegetable oils was not significantly different ($p>0.05$). All oils tested were comprised mainly of three fatty acids, oleic acid, linoleic acid and palmitic acid, in varying proportions. Oleic acid and linoleic acid are unsaturated fatty acids, whereas palmitic acid and stearic acid are saturated fatty acids (Patil and Chopade, 2001). According to olive oil, corn oil and sunflower oil display a higher degree of

unsaturation as compared with palm oil that has the least degree of unsaturation. Olive oil is rich in unsaturated oleic acid ($C_{18:1}$), suggesting a high emulsification specificity with the spent yeast bioemulsifier.

The spent yeast bioemulsifier exhibited emulsification activity higher than the commercial emulsifiers such as gum arabic and lecithin. Gum arabic could not emulsify palm oil and sesame oil. Lecithin could not emulsify sesame oil and corn oil. The stability of the emulsions were affected by the composition of the oil dispersed phase as found by Driscoll *et al.* (2001). Moreover, structure of three compounds (the spent yeast bioemulsifier, gum arabic and lecithin) was also considered. The structure of gum arabic is more similar to the spent yeast bioemulsifier than lecithin. The structure of gum arabic is composed of a highly branched arrangement of the simple sugars galactose, arabinose, rhamnose, and glucuronic acids. It also contains a protein component (about 2 percent, w/w) covalently bound within its molecular arrangement (McNamee *et al*., 1998). Thus, gum arabic consisted of carbohydrate and a small amount of protein like the spent yeast bioemulsifier. On the other hand, lecithin contains phospholipids. It is composed of glycerol, two fatty acid, phosphate and has a nitrogenous base (Hasenhuettl and Hartel, 2008). Therefore, the emulsification activity of the spent yeast bioemulsifier was much more similar to gum arabic than lecithin.

Table 9. Vegetable oil emulsification by the spent yeast bioemulsifier, gum arabic and lecithin.

Oil type	Bioemulsifier*	Gum arabic	Lecithin
Olive oil	$62.67 \pm 1.77^{A**a***}$	65.52 ± 0.00^{Aa}	54.28 ± 2.67^{Cb}
Soybean oil	58.17 ± 2.80^{CDa}	61.38 ± 1.20^{Ba}	59.52 ± 2.06^{Ba}
Palm oil	60.74 ± 1.96^{ABCb}	0.00 ± 0.00^{Cc}	64.20 ± 2.14^{Aa}
Rice bran oil	58.55 ± 1.22^{BCDa}	58.13 ± 3.55^{Ba}	61.42 ± 2.59^{ABa}
Sesame oil	55.83 ± 1.14^{Da}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Db}
Corn oil	62.03 ± 1.31^{Aa}	61.64 ± 2.62^{Ba}	0.00 ± 0.00^{Db}
Sunflower oil	61.62 ± 0.78^{ABa}	60.01 ± 1.21^{Ba}	55.52 ± 1.64^{Cb}

* Values are given as mean ± SD from triplicate determinations.

** Different capital letters in the same column indicate significant differences ($p<0.05$).

*** Different small letters in the same row indicate significant differences ($p<0.05$).

2.4.3 Stability of the spent yeast bioemulsifier

The effects of various pHs (3-12) on the emulsion activity of the spent yeast bioemulsifier are presented in Figure 9. The emulsion activity(emulsification activity and emulsification index) of the spent yeast bioemulsifier decreased clearly with decreasing pH below 5 due to precipitation of the bioemulsifier (Kim *et al*., 1997 and Sutthivanichakul *et al*., 1999). However, no changes in activity were noticeable in the pH range of pH 5-12.

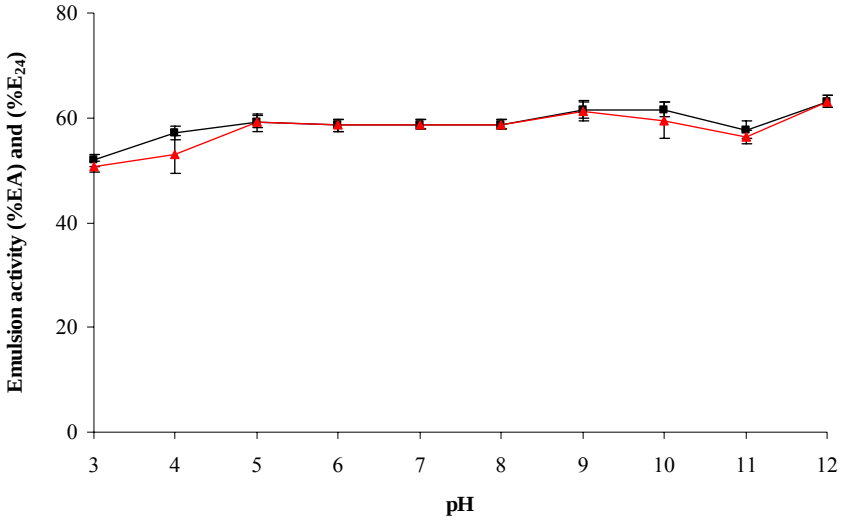


Figure 9. Effect of pH on emulsion activity of the crude spent yeast bioemulsifier. ■: emulsification activity (%EA), ▲: emulsification index ($\%E_{24}$). Bars represent the standard deviation from triplicate.

After the spent yeast bioemulsifier was incubated at temperatures which are usually used in food processing such as pasteurization, cooking and food thermal processing, the residual emulsion activity was determined (Table 10). All the temperatures tested did not show any influence on the emulsion activity (emulsification activity and emulsification index) towards palm oil of the spent yeast bioemulsifier. This characteristic of the spent yeast bioemulsifier may be attributed to certain chemical groups that endow it with protection from hydrolytic degradation. This is a useful property for many commercial applications that involve surface-active or emulsifying agents in formulations subjected to high temperature treatments (Gutierrez *et al*., 2009). Moreover, temperature storage condition at 4ºC, 10ºC and

room temperature had no effect on stability of emulsion, 4°C was the best temperature storage for stability studied. (Table 11).

Table 10. Effect of temperatures on stability of the spent yeast bioemulsifier.

Temperature (°C)	Holding time (min)	Emulsification activity (%EA)	Emulsification index* ($\%E_{24}$)
63	30	$60.98 \pm 1.86^{a**}$	$60.98 \pm 1.86^{a**}$
100	15	61.54 ± 2.07^{a}	59.50 ± 1.94^{a}
121	15	61.54 ± 2.22^{a}	60.26 ± 2.22^{a}

* Values are given as mean ± SD from triplicate determinations.

** Different letter in the same column indicate significant differences ($p<0.05$).

Table 11. Effect of temperatures on stability of the emulsion formed between palm oil and the spent yeast bioemulsifier

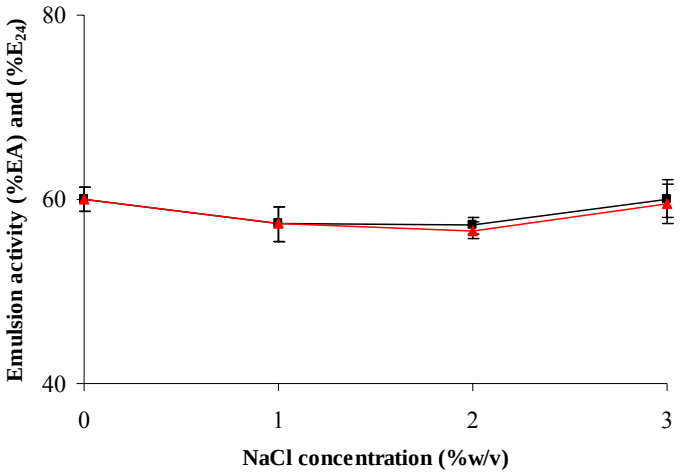
Temperature (ºC)	Holding time (h)	Emulsification activity (%EA)	Emulsification index* ($\%E_{24}$)
room temperature	24	$61.54 \pm 1.28^{b**}$	$60.02 \pm 1.32^{b**}$
4	24	64.47 ± 2.07^{a}	64.47 ± 2.07^{a}
10	24	61.78 ± 3.20^{ab}	61.78 ± 3.20^{ab}

* Values are given as mean ± SD from triplicate determinations.

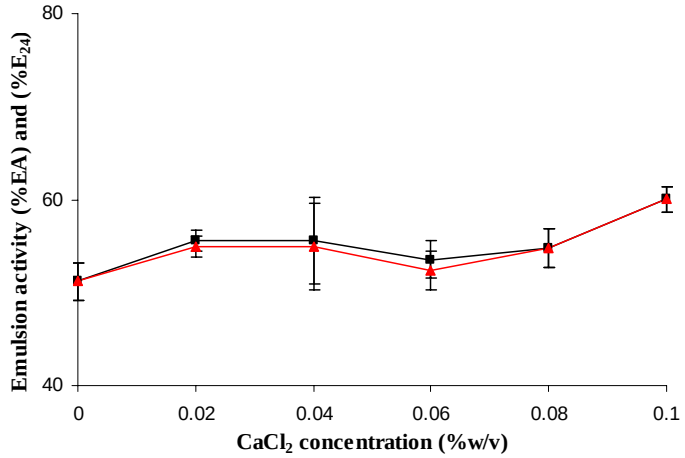
** Different letter in the same column indicate significant differences ($p<0.05$).

The effect of salts on the emulsion activity (emulsification activity and emulsification index) of the spent yeast bioemulsifier is illustrated in Figure 10. NaCl and $MgCl_2$ had no effect on the emulsion activity of the spent yeast bioemulsifier. However, the addition of more $MgCl_2$ change to the bigger droplet of emulsion while remain had stability of the emulsion. A slight decrease in activity of the spent yeast bioemulsifier was observed when $CaCl_2$ was added. These were emulsions that were unstable at a higher $CaCl_2$ concentration. The addition of more $CaCl_2$ might destabilize emulsions through reducing the electrostatic repulsion among droplets (Klinkesorn and Namatsila, 2009).

a



b



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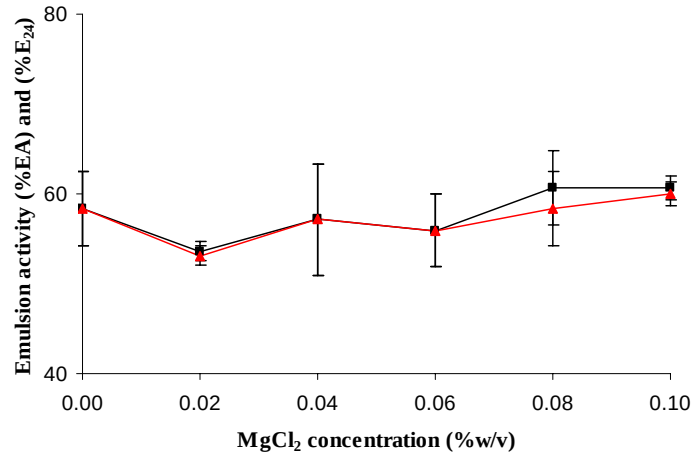


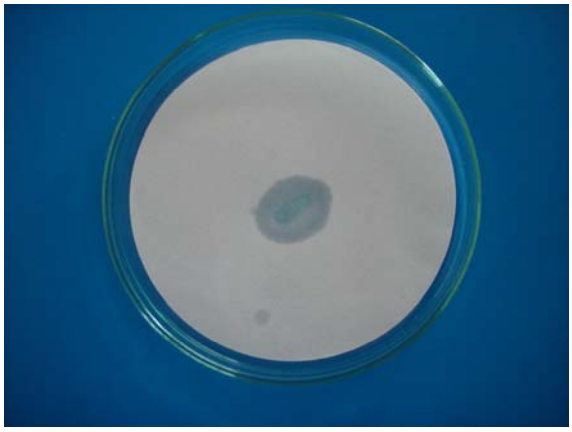
Figure 10. Effects of NaCl (a), $CaCl_2$ (b) and $MgCl_2$ (c) concentration on emulsion activity of the crude spent yeast bioemulsifier. ■: emulsification activity (%EA), ▲: emulsification index ($\%E_{24}$). Bars represent the standard deviation from triplicate determinations.

The bioemulsifier extracted from spent yeast obtained from traditional liquor distillation was stable over a wide range of physical and chemical conditions. The results were in accordance with the previous studies that the bioemulsifiers from *S. cerevisiae* and *Kluyveromyces marxianus* FII 510700 was stable in a wide rang of pHs, temperatures and salt concentration (Torabizadeh *et al*., 1996; Lukondeh *et al*., 2003).

2.4.3 Determination of emulsion type and droplet size distribution

The spent yeast bioemulsifier promoted the formation of oil-in-water emulsion. Because such emulsions dispersed rapidly on filter paper but cloudily in water, and remained a droplet in oil (Fig. 11). Base on the nature of the dispersed phase, macroemulsions are usually one of the two types. Oil in water (o/w) or water in oil (w/o). However, the type of emulsion formed depends primarily on the nature of the emulsifier and on the ratio of components involved and method emulsification. Oil in water emulsions are produced by emulsifying agents that are more soluble in the water phase than in the oil phase, whereas water in oil emulsions are produced using emulsifying agents that are more soluble in the oil phase than in the aqueous phase (Rhein, 2007).

(a)



(b-1)

(b-2)

Figure 11. Determination of type of emulsion formed (a) filter paper wetting test and (b-1) dilution test of droplet in oil (b-2) dilution test of droplet in water.

The droplet mean diameters of d(10), d(50), d(90) and Span are summarized in Table 12. It is generally important that emulsion droplets are made as small as possible. A convenient way to evaluate the relative effectiveness of an emulsifier is to determine droplet size distribution. The droplet size of the spent yeast bioemulsifier was compared with commercial emulsifiers (gum arabic and lecithin). There were 3 pieces of information on distribution. d(10), d(50) and d(90) showed that there are about 10%, 50% and 90% of smaller droplets (μm) in the distribution, respectively.

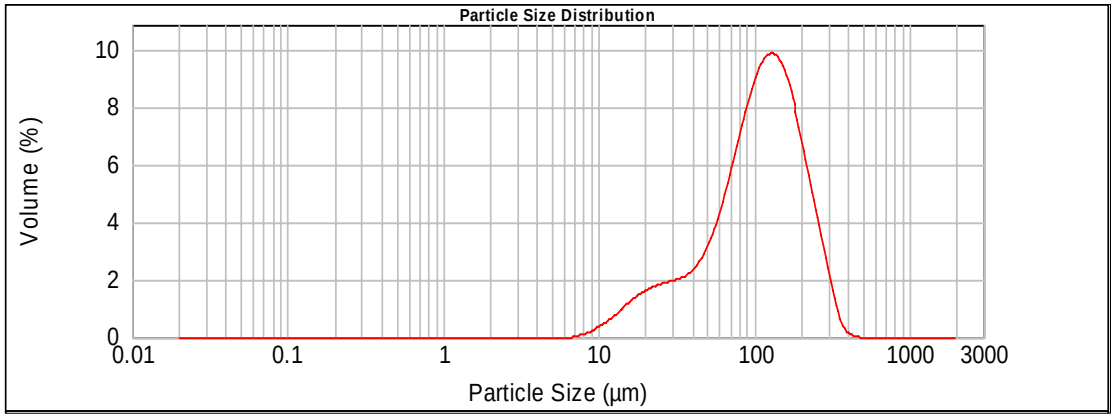
Table 12. Droplet mean diameters and dispersity index (span) of emulsions.

Samples	d(10)* μm	d(50)* μm	d(90)* μm	Span
Spent yeast bioemulsifier	30.897	110.836	224.124	1.74
Gum arabic	35.676	105.670	189.049	1.45
Lecithin	26.449	86.464	170.747	1.67

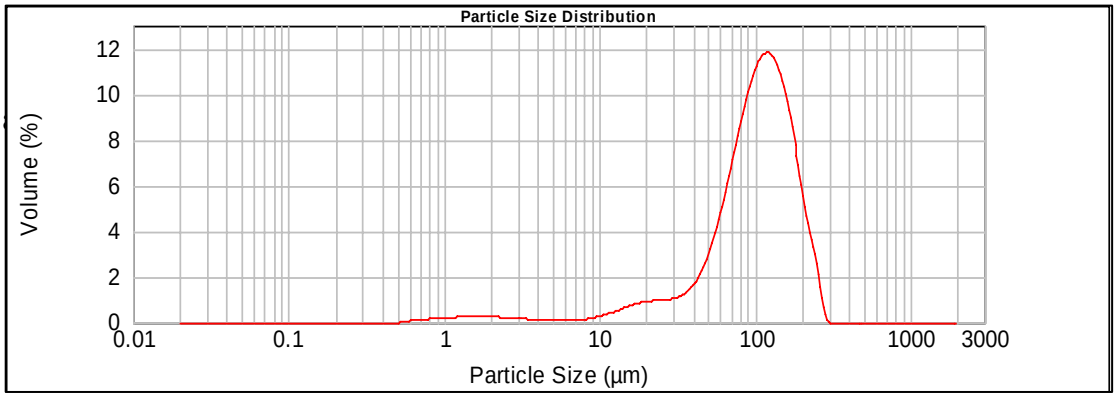
* The experiments were done in triplicate and results were reported as the average from triplicate determinations.

The span indicates the width of the distribution regardless of the median size (Palazolo *et al*., 2004). Vegetable oil, which gives the highest emulsion with each emulsifier, was used for the investigation. The particle size where the cumulative distribution is 50% is known as the median droplet diameter ($d_{v,0.5}$). All emulsions showed a monomodal distribution of droplets. Emulsion from the spent yeast bioemulsifier had a $d_{v,0.5}$ with 50% of the particles under 110.836 μm, compared with emulsion from gum arabic and lecithin which had 105.670 μm and 86.464 μm, respectively (Fig. 12). It revealed that the emulsion from lecithin had the smallest particle size. However, the span of lecithin emulsion was not the lowest. The span of gum arabic was the lowest, indicating that it had the lowest polydispersity of the emulsion. It showed more stability of emulsion than others. Particle size distribution is a key characteristic, as it contributes to the physical stability property of the emulsion. Thus, obtaining an emulsion with a uniformly smaller droplet size becomes essential to achieve a stable emulsion system. As a rule, large globules tend to coalesce faster than small ones (McClements, 1999).

a



b



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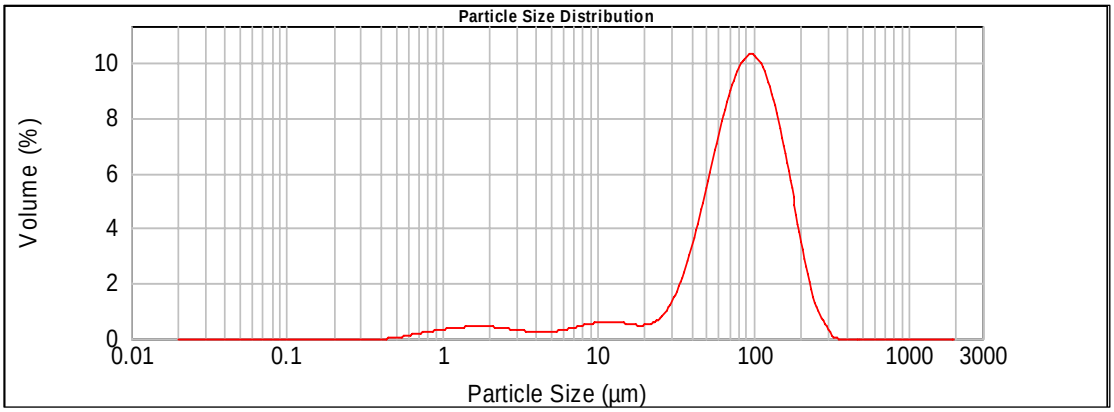


Figure 12. Particle size distribution of emulsion droplets of the spent yeast bioemulsifier by using palm oil as a substrate for determined emulsion activity (a), gum arabic by using olive oil as a substrate for determined emulsion activity (b), and (c) lecithin by using palm oil as a substrate for determined emulsion activity.

2.5 Conclusion

The spent yeast produced as a waste from the local liquor distillation could provide a source of raw material for the mass production of the spent yeast bioemulsifier. This could eliminate the need to grow the yeast specifically for the production of emulsifier. Besides, the spent yeast bioemulsifier can extract by the low-cost method, using water-soluble substrates (citrate buffer). As a result, this extraction procedure is simple and suitable for large-scale production and utilizing the abundant waste from distillery.

CHAPTER 3

BIOEMULSIFIER OF YEAST ISOLATED FROM THAI TRADITIONAL LIQUOR: EXTRACTION AND CHARACTERIZATION

3.1 Abstract

Saccharomyces cerevisiae KA01 was isolated from sugar palm wine obtained from a local brewer in Songkhla Province, Thailand. Yeast bioemulsifier was extracted from the cell walls of *S. cerevisiae* KA01 cultivated in YM broth by autoclaving in a neutral citrate buffer pH 7.0 at 121°C under pressure of 15 psi for 60 min. The yeast bioemulsifier obtained was evaluated for its chemical and physical stability in order to establish its potential use as a natural emulsifier in processed foods. The yield of yeast bioemulsifier was 0.32 g/g wet cells. Extracted yeast bioemulsifier exhibited emulsion with the vegetable oils tested. It showed emulsion activity of 65% toward palm oil as oil-in-water and had a critical emulsifier concentration of 20 g/l. Extracted yeast bioemulsifier had emulsifying properties as good as those of the commonly used food emulsifiers gum arabic and lecithin. Palm oil-in-water emulsion was stabilized over a broad range of conditions, from pH 5 to 8, with up to 3% (w/v) sodium chloride, and up to 0.1% (w/v) $CaCl_2$ and $MgCl_2$ in the aqueous phase. Temperatures (4°C, 10°C, room temperature, 63°C, 100°C, and 121°C) did not affect the emulsion activity of yeast bioemulsifier. Preliminary trials showed that the extracted yeast bioemulsifier from *S. cerevisiae* KA01 had potential for use as emulsifier in salad dressing because emulsion of the yeast bioemulsifier showed longer stability than commercial emulsifier.

3.2 Introduction

Surfactants and emulsifiers are amphipathic molecules. They have both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces (Vance *et al*., 2003). As a result, surfactants and emulsifiers reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surfactants and emulsifiers integral to many industrial, agricultural and food processes. Mannoprotein has been shown to be an effective bioemulsifier. The presence of hydrophilic mannose polymers covalently attached to the protein backbone provides the mannoprotein with the amphiphilic structure common to surface active agents and effective emulsifier (Cameron *et al*., 1988).

Despite their typically high production costs, emulsifiers derived from natural sources may have certain advantages over the chemically synthesized emulsifying agents. Due to the increasing consumer demand for natural products, bioemulsifiers may eventually become cost-effective for various applications. Some natural, plant-derived, food emulsifiers such as lecithin and gum arabic are already on the market. However, these emulsifiers have limited functionality in many food products (Shepherd *et al*., 1995). The production of food emulsifiers by microbial cultivation would remove some of the constraints associated with the properties and supply of natural, plant-derived emulsifiers.

Saccharomyces cerevisiae is generally used for alcohol fermentation. Mannoprotein extracted from *S. cerevisiae* has been shown to be an effective bioemulsifier (Cameron *et al*., 1988; Torabizadeh *et al*., 1996). The emulsions produced are thick and viscous. Since *S. cerevisiae* is edible and is used in food and beverage products, the emulsifier would be expected to be nontoxic. An emulsifier with these properties would have applications in the food and cosmetics industries. This product also satisfies the current consumer demand for natural and environmentally safe products. Mannoprotein are freely soluble in water and can be extracted from the cell wall of *S. cerevisiae* in high yields (Ballou, 1976; Cabib and

Roberts, 1982; Cameron *et al*., 1988). Thus, strains of *S. cerevisiae* produced by low-cost biotechnology methods using water-soluble substrates, as well as brewing industries, have become important sources from which bioemulsifiers are extracted (Barriga *et al*., 1999; Torabizadeh *et al*., 1996). These sources offer the advantages of low cost and a high volume of yeast biomass, which translates into high bioemulsifier yields in comparison with synthetic sources.

The objectives of this study were to extract and characterize bioemulsifier from yeast isolated from palm sugar wine obtained from local brewer in Songkhla Province, Thailand. These is would be compared with the characteristics of commercial bioemulsifiers, lecithin and gum arabic. The potential of extracted bioemulsifier for use in salad dressing was also investigated.

3.3 Materials and Methods

3.3.1 Chemicals

Commercial vegetable oils (soybean oil, palm oil, corn oil, olive oil, sunflower oil, rice bran oil and sesame oil) were purchased from a supermarket. Gum arabic was purchased from Nacalai Tesgue, Inc. (Kyoto, Japan). Lecithin was obtained from Fluka (USA). All other chemicals used were of analytical grade.

3.3.2 Yeast strain, medium and growth conditions

Palm sugar wine at the final stage of fermentation was collected from a local brewer at Singhanakorn District, Songkhla Province, Thailand. Direct isolation was performed by serially diluting samples in 0.85% sterile normal saline, and a sample dilution from 10^{-1} to 10^{-7} was prepared. Aliquots were spread-plated on the YM agar (containing malt extract powder (Hi-media, Thailand), yeast extract (Lab-scan, Thailand) and peptone bacteriological (Hi-media, Thailand)) and incubated at room temperature for 48 h. Morphologically distinct yeast colonies were randomly selected and streaked on YM agar. This procedure was repeated in order to purify the isolates. The isolated yeast was maintained on YM slants at 4°C and transferred at 1 month intervals. The isolated yeast strain was identified based on the 26S rDNA sequence analysis.

Saccharomyces cerevisiae KA01 was grown in 200 ml of YM broth in 500 ml flasks. The flasks were incubated at room temperature for 12 h (early stationary phase) at 150 rpm.

3.3.3 Extraction and semi-purification of yeast fraction enriched in bioemulsifier

Bioemulsifier was extracted from cells of *S. cerevisiae* KA01 by the method of Torabizadeh *et al*. (1996). Yeast cells (20 g) were suspended in 100 ml. 0.1 M potassium citrate and 0.02 M potassium metabisulfite buffer, pH 7, and autoclaved (121ºC) for various periods (15-120 min). The resulting suspensions were centrifuged at 6,000×*g* for 10 min at 4ºC. The supernatant was retained and mixed with five volumes of chilled ethanol, incubated overnight at 4ºC for complete precipitation. The precipitates were recovered by centrifugation at 6,000×*g* for 10 min at 4ºC, and then washed twice with chilled ethanol. The precipitates were freeze-dried for 48 h. Crude yeast bioemulsifier was dissolved in distilled water and dialyzed (8,000 dalton molecular weight cut-off) against distilled water overnight. The yeast bioemulsifier was then freeze-dried. Total protein (Bradford, 1976) and total sugar (Dubois *et al*., 1956) were measured.

3.3.4 Determination of type of emulsion formed

A filter paper wetting test and a dilution test (Rieger, 1986) were used to determine the type of emulsion formed. For the filter paper wetting test, droplet of emulsion was dropped onto filter paper. If the emulsion is water-in-oil (w/o), droplet of emulsion would remain a droplet on the filter paper. If the emulsion is oil-in-water (o/w) droplet of emulsion would disperse rapidly on the filter paper. For the dilution test, droplet of emulsion was dropped into water and oil. If the emulsion is w/o, droplet of emulsion would disperse in the oil and remain a droplet in the water. If the emulsion is o/w, droplet of emulsion would disperse cloudy in the water and remain a droplet in the oil.

3.3.5 Stability of fraction enriched in the yeast bioemulsifier

The yeast bioemulsifier (20 g/l) was prepared in distilled water. To investigate the effects of pH, salts concentration (NaCl, $CaCl_2$ and $MgCl_2$) and temperature on emulsion activity (emulsification activity and emulsification index) of the yeast bioemulsifier, the yeast bioemulsifier solution was adjusted with 1 N HCl or NaOH to obtain a pHs of 3-12. NaCl was added to the sample to obtain the final concentrations of 0-3% (w/v). $CaCl_2$ and $MgCl_2$ were also added to the samples to obtain the final concentrations of 0-0.1% (w/v). For the study of thermal stability, the yeast bioemulsifier solution was incubated at 63ºC for 30 min, at 100ºC for 15 min and at 121ºC for 15 min and cooled to 30ºC. The remaining activity was then determined. Commercial bioemulsifiers (lecithin and gum arabic) at the same concentrations were also subjected to a stability study.

3.3.6 Determination of droplet size distribution

The droplet size distributions (DSD) for fresh emulsions were measured approximately 1 h after the preparation by a laser diffraction method as described by Hayati *et al*. (2007). Distilled water was used as the dispersant for the determination of the emulsion lipid globule size distribution. The software used a reflective index of dispersant RI 1.33 (water) to calculate the Dispersion Index (Span) by span = d(90)-d(10)/d(50). The d(10), d(50) and d(90) values are size values corresponding to the cumulative distribution at 10%, 50% and 90%, respectively. Thus the d(10) represents a size value below which 10% of the cumulative distribution is present. Drops of emulsion were introduced into the sample presentation unit until the concentration reached the optimum one, as indicated by the instrument.

3.3.7 Preparation of salad dressing

The salad dressing formulation consisted of soybean oil 24.39% (w/w), vinegar 54.20% (w/w), sugar 14.09% (w/w), table salt 4.34% (w/w), pepper 1.63% (w/w) and mustard 1.35% (w/w) and with or without bioemulsifiers (yeast bioemulsifier, gum arabic or lecithin, 0.2-0.6%, w/w). The dry ingredients were mixed ,vinegar was added when the dry ingredients were dispersed, and the soybean oil was gradually incorporated into the mixture. The mixture was mixed by vortex at the highest speed.

3.3.8 Analytical methods

The total carbohydrate was determined colorimetrically by the method of Dubois *et al*. (1956) with mannose used as the standard. Protein content was determined using the dye-binding assay (Bradford, 1976) with bovine serum albumin used as the standard. The average molecular weight of semi-purified yeast bioemulsifier was determined by gel permeation chromatography (GPC). The analysis was carried out using Waters 2410, 600E fraction collector II, Waters Division of MILLIPORE (preparative system). The universal calibration was obtained by using pullulan standards with molecular weights ranging from 5,900 to 788,000 Dalton. Semi-purified bioemulsifier (2 mg/ml) from isolated yeast was dissolved in 0.05M sodium bicarbonate buffer pH 11 and filtered using nylon 66 membrane (pore size 0.45 μm) before injection into gel permeation chromatography. Emulsion activity (emulsification activity and emulsification index) was measured according to the method of Cameron *et al*. (1988) but slightly modified. One ml of vegetable oil was added to one ml of the yeast bioemulsifier suspension and vortexed at high speed for 3 min. The mixture was allowed to stand for 1 h (emulsification activity, %EA) and 24 h (emulsification index, $\%E_{24}$) prior to measurement. Emulsification activity (%EA) or emulsification index, $\%E_{24}$) is defined as the height of the emulsion layer divided by the total height and expressed as percentage. (%EA) is refer only activity whereas ($\%E_{24}$) involve both activity and stability after stay for 24 h. The experiments were done in triplicate and results were reported as the average from the determinations in triplicate.

3.3.9 Statistical analysis

Data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan's multiple-range test. Statistical analysis was performed using the Statistical Package for Science (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL).

3.4 Results and Discussion

3.4.1 Isolation of yeast

Generally, Thai traditional spirit producers produce wine (rice wine, palm sugar wine) in earthenware using indigenous microorganisms. They may also use the back-slopping technique to produce wine. In the present study palm sugar wine in the last stage of fermentation before subjection to the distillation process was used as a source of microorganisms. Appropriate dilution samples were spread-plated on YM agar and randomly picked as 5-10 colonies from each plate. It revealed that only one yeast strain dominated in the palm sugar wine at the last fermentation stage. The isolated yeast strain produced an off-white colored, smooth, raised and glistening colony. The morphological features of the isolated yeast under microscope were oval shape and multipolar budding (Fig. 13). It also had 2-4 ascospores when cultivated in ascospore-inducing medium (acetate agar) see Fig. 14. Based on 26S rDNA sequence analysis, the isolated yeast was identified as *Saccharomyces cerevisiae* with 99% homology (505 bp). The rDNA sequence was deposited in DDBJ/EMBL/GenBank as accession number AB510531.

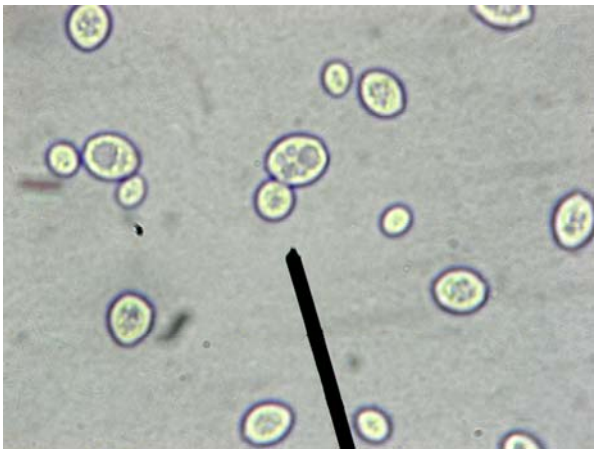


Figure 13. Morphological of the isolated yeast strain (*Saccharomyces cerevisiae* KA01) under a compound microscope (× 400 magnification).

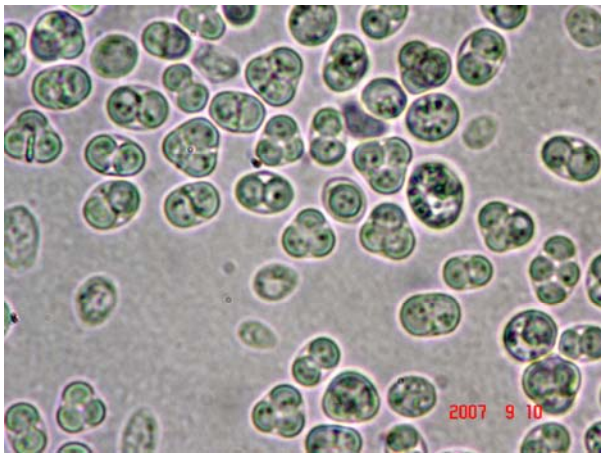


Figure 14. Ascospore reproduction of the isolated yeast strain (*Saccharomyces cerevisiae* KA01) in a compound microscope (× 400 magnification) after incubated at room temperature for 21 days on acetate agar.

In the production of wine, natural fruit juice fermentation is carried out by a succession of different yeast populations. Several authors report that the early stages of the alcoholic fermentation are characterized by the activity of mostly apiculate, non-*Saccharomyces* yeasts from *Kloeckera* and *Hanseniaspora* genera (Charoenchai *et al*., 1998; Gil *et al*., 1996; Gutierrez *et al*., 1999; Romano *et al*., 1997). The growth of these yeasts is generally limited to the first two or three days of fermentation, after which they die off, giving way to the more tolerant strains of *Saccharomyces cerevisiae* (Cianni and Picciotti, 1995; Fleet, 1990; Gil *et al*., 1996; Martinez *et al*., 1989). Many physiological parameters allow *Saccharomyces cerevisiae* to dominate fruit juice fermentations. Its tolerance to high concentrations of ethanol is the principal feature of this yeast that allows its survival in this specific environment (Boulton *et al*., 1995).

3.4.2 Extraction and semi-purification of the yeast bioemulsifier

The effect of extraction time on emulsifier yield, emulsification index ($\%E_{24}$) and critical emulsifier concentration of the yeast bioemulsifier from *S. cerevisiae* KA01 are summarized in Table 13. It was found that the yield of the yeast bioemulsifier increased with increasing extraction time. Critical emulsifier concentration of all extraction time was 20 g/l. The effectiveness of the bioemulsifer is evident from its low critical emulsifier concentration (Barriga *et al*., 1999; Mohana *et*

al., 2009). Nevertheless, autoclaving for 15 min gave the lowest the yeast bioemulsifier yield and emulsification activity value. In addition, it also gave less stability of emulsion toward palm oil after being left to stand at room temperature for many days (data not shown). Although autoclaving for 30 min brought critical emulsifier concentration 20 g/l, it gave less stability that notice from the big droplet size of the emulsion. Emulsifier yield, emulsification index and critical emulsifier concentration of emulsifiers from extraction time at 60, 90 and 120 min showed no significant difference ($p<0.05$). Accordingly, autoclaving for 60 minutes was the best condition for bioemulsifier extraction from *S. cerevisiae* KA01 in terms of cost and energy consumption. In the present study extraction time was shorter than Barriga *et al*. (1999); Cameron *et al*. (1988); Torabizadeh *et al*. (1996) who found that the optimum autoclaving time for bioemulsifier extraction from *S. cerevisiae* was 120 minutes.

Table 13. Effect of extraction time by heating at 121°C under pressure of 15 psi on the yeast bioemulsifier yield, emulsification index ($\%E_{24}$) and critical emulsifier concentration.

Holding time in autoclave (min)	Bioemulsifier yield[a] (g/g dry cell weight)	Emulsification index($\%E_{24}$)	Critical emulsifier concentration (g/l)
15	$0.580 \pm 0.009^{d**}$	48.97 ± 3.98^{b}	20
30	0.629 ± 0.008^{c}	63.40 ± 0.77^{a}	20
60	0.644 ± 0.022^{bc}	64.70 ± 0.71^{a}	20
90	0.677 ± 0.016^{a}	64.29 ± 0.00^{a}	20
120	0.666 ± 0.003^{ab}	64.29 ± 0.00^{a}	20

[a] Values are given as mean ± SD from triplicate determinations.

** Different letter in the same column indicate significant differences ($p<0.05$).

The apparent molecular weight of the semi-purified yeast bioemulsifier, when compared with that of pullulan standards, was 76 kDa (Fig. 15). The composition of the yeast bioemulsifier was 58.37% carbohydrate and 41.63% protein.

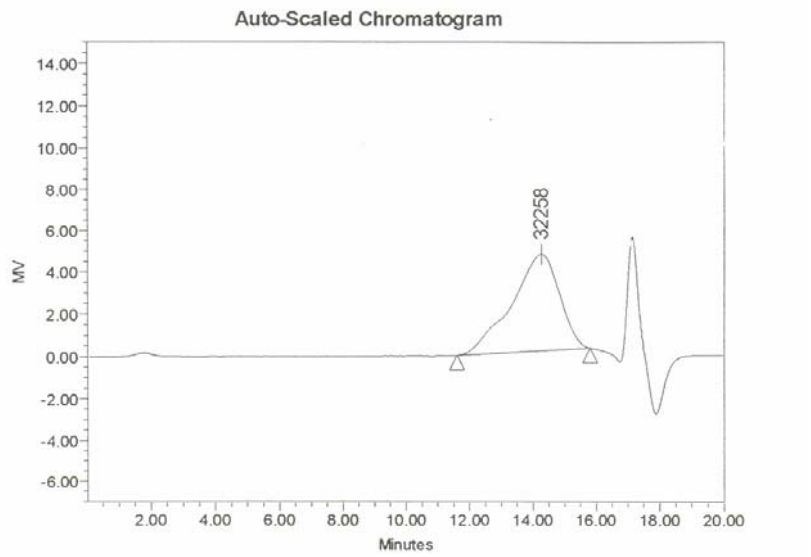


Figure 15. GPC chromatogram of the semi-purified yeast bioemulsifier.

3.4.3 Stability of the yeast bioemulsifier

The yeast bioemulsifier, gum arabic and lecithin at the critical emulsifier concentration (20 g/l) were checked for specificity of vegetable oil emulsion. All the vegetable oils tested, except rice bran oil, served as substrates for emulsification by the yeast bioemulsifier (Table 14). Poor emulsification of some vegetable oils, rice bran oil might be due to the inability of the yeast bioemulsifier to stabilize the microscopic droplets. Maximum emulsifying activity was observed with palm oil; however, the emulsification index of the yeast bioemulsifier toward palm oil and olive oil were not significantly different ($p>0.05$). All the tested oils comprise mainly of three fatty acids, oleic acid, linoleic acid and palmitic acid, in varying proportions. Oleic acid and linoleic acid are unsaturated fatty acids, whereas palmitic acid is a saturated fatty acid (Patil and Chopade, 2001). Accordingly, palm oil display a lower degree of unsaturation as compared to olive oil, with is rich in unsaturated oleic acid ($C_{18:1}$), Perhaps the yeast bioemulsifier has more specificity with palmitic acid, which is the main composition of palm oil, than oleic and linoleic acid. So, this suggests that palm oil showed high emulsification specificity with the yeast

bioemulsifier. Studies of specificity for substrates have indicated that yeast bioemulsifier is capable of forming stable emulsions with various vegetable oils, as it can be used as an emulsifying agent for these compounds. The capacity to form emulsions with vegetable oils suggests potential applications as an emulsifying agent in the food industry.

The yeast bioemulsifier can emulsified more variety vegetable oil than the commercial emulsifiers, gum arabic and lecithin. Gum arabic could not emulsify palm oil and sesame oil. Lecithin could not emulsify sesame oil and corn oil. The emulsion can of course coalesce if different fatty acid globules of each vegetable oil were destabilized and the interfacial layer was weak enough. The stability of the emulsions will be affected by the composition of the oil dispersed phase in accordance to the finding of Driscoll *et al*. (2001). Moreover, the structure of three compounds (the yeast bioemulsifier, gum arabic and lecithin) was also considered. The structure of gum arabic is more similar to the yeast bioemulsifier than lecithin. The structure of gum arabic is composed of a highly branched arrangement of the simple sugars galactose, arabinose, rhamnose, and glucuronic acids. This also contains a protein component (about 2 percent, w/w) covalently bound within its molecular arrangement (Mcnamee *et al*., 1998). Thus, gum arabic consisted of carbohydrate and a small amount of protein like bioemulsifier. On the other hand, lecithin is made up of phospholipids. It is composed of glycerol, two fatty acids, and phosphate and has a nitrogenous base. Accordingly, in term of chemical composition the emulsification activity of the yeast bioemulsifier was much more similar to gum arabic than lecithin.

Table 14. Vegetable oil emulsification by the yeast bioemulsifier, gum arabic and lecithin ($\%E_{24}$).

Oil type	yeast bioemulsifier*	Gum arabic	Lecithin
Olive oil	$64.69 \pm 0.72^{AB**a***}$	65.52 ± 0.00^{Aa}	54.28 ± 2.67^{Cb}
Soybean oil	56.98 ± 1.73^{Cb}	61.38 ± 1.20^{Ba}	59.52 ± 2.06^{Bab}
Palm oil	65.49 ± 1.19^{Aa}	0.00 ± 0.00^{Cb}	64.20 ± 2.14^{Aa}
Rice bran oil	0.00 ± 0.00^{Db}	58.13 ± 3.55^{Ba}	61.42 ± 2.59^{ABa}
Sesame oil	57.63 ± 0.85^{Ca}	0.00 ± 0.00^{Cb}	0.00 ± 0.00^{Db}
Corn oil	57.14 ± 0.00^{Cb}	61.64 ± 2.62^{Ba}	0.00 ± 0.00^{Dc}
Sunflower oil	62.77 ± 2.48^{Ba}	60.01 ± 1.21^{Ba}	55.52 ± 1.64^{Cb}

* Values are given as mean ± SD from triplicate determinations.

** Different capital letters in the same column indicate significant differences ($p<0.05$).

*** Different lowercase letters in the same row indicate significant differences ($p<0.05$).

The effects of various pHs (3-12) on the emulsion activity (emulsification activity and emulsification index) of the yeast bioemulsifier are presented in Figure 16. The yeast bioemulsifier was the most active in the pH range of 5-8. A slight decrease in emulsion activity was observed at the pH lower than 5 due to the precipitation of the yeast bioemulsifier. On the other hand the emulsion activity was obviously decreased with an increasing pH higher than 9. Different emulsifiers are known to function of different optimal pH levels.

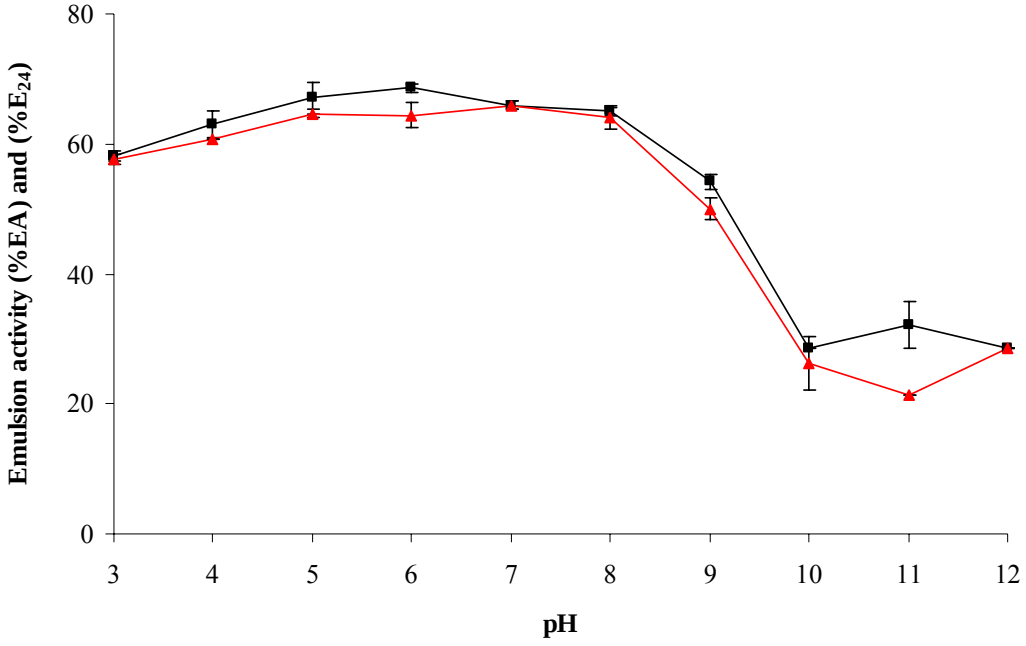


Figure 16. Effect of pH on emulsion activity of the yeast bioemulsifier. ■: emulsification activity (%EA), ▲: emulsification index ($\%E_{24}$). Bars represent the standard deviation from triplicate.

The stability of the yeast bioemulsifier was tested over a wide temperature range. Study on the effect of heat treatment demonstrated that heating methods which are usually use in food processing, such as pasteurization, cooking and food thermal processing, have no effect on emulsion activity (emulsification activity and emulsification index) (Table 15). Each temperature (63°C, 100°C, and 121°C) tested showed no influence on the stability toward palm oil of the yeast bioemulsifier. This feature of the yeast bioemulsifier may be attributed to certain chemical groups that endow it with protection from hydrolytic degradation (Gutierrez *et al*., 2009). This is a useful property for the many commercial applications that involve surface-active or emulsifying agents in formulations subjected to high temperature treatments. The stability of the yeast bioemulsifier in relation to temperature indicates the usefulness of the yeast bioemulsifier in industries where heating to achieve sterility is of paramount importance.

Table 15. Effect of temperatures on stability of the yeast bioemulsifier.

Temperature (°C)	Holding time (min)	Emulsification activity* (%EA)	Emulsification index* ($\%E_{24}$)
63	30	$65.11 \pm 0.72^{a**}$	$65.11 \pm 0.72^{a**}$
100	15	65.11 ± 0.72^{a}	65.11 ± 0.72^{a}
121	15	65.11 ± 0.72^{a}	63.54 ± 1.28^{a}

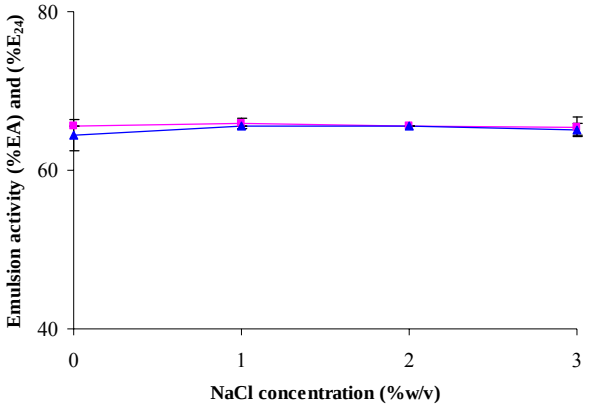
* Values are given as mean ± SD from triplicate determinations.

** Different letter in the same column indicate significant differences ($p < 0.05$).

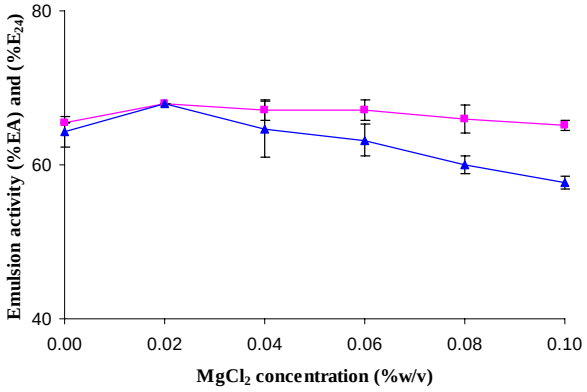
Experiments were performed to examine the influence of NaCl, $MgCl_2$ and $CaCl_2$ concentration on the emulsion activity (emulsification activity and emulsification index). The effect of salts on the emulsion activity of the yeast bioemulsifier is illustrated in Figure 17. The emulsion activity against vegetable oil remained practically unchanged in the concentrations of NaCl and $MgCl_2$ tested. NaCl and $MgCl_2$ had no effect on the activity of the yeast bioemulsifier. However, a decrease in the emulsion activity of the yeast bioemulsifier was observed when $CaCl_2$ was added. When increasing the concentration to a higher $CaCl_2$, the emulsification activity (%EA) remained stable whereas the emulsification index ($\%E_{24}$) decreased until it showed 20% at 0.1% (w/v) of $CaCl_2$ concentration. Because emulsions unstable at higher $CaCl_2$ concentration. The addition of $CaCl_2$ might destabilize emulsions through reducing the electrostatic repulsion among droplets because the small ion of Ca^{2+} alter the conformation of the adsorbed protein layer. (Klinkesorn and Namatsila, 2009). The stability of the yeast bioemulsifier to salinity suggested that it is a good candidate for use in some industries that involve emulsions. This feature would be of great interest in food production that has highly salted food. The bioemulsifier extracted from yeast isolated from traditional liquor distillation was stable over a wide range of physical and chemical conditions and showed certain emulsification properties. These findings revealed that the product obtained could be very useful in situations where extreme conditions of temperature, salinity and pH 5-8 are present. The results were in accordance with previous studies that bioemulsifier from *S. cerevisiae* and *Kluyveromyces marxianus* FII 510700 were stable in a wide range of pHs, temperatures and salts Emulsions of the bioemulsifier from *S. cerevisiae* was

stable in a range of pH (3-11) and NaCl concentration (5-50 g/l). Also, emulsions of the bioemulsifier from *K. marxianus* was stable in a range of pH (3-11) and NaCl concentration (2-50 g/l) (Torabizadeh *et al*., 1996; Lukondeh *et al*., 2003).

(a)



(b)



(c)

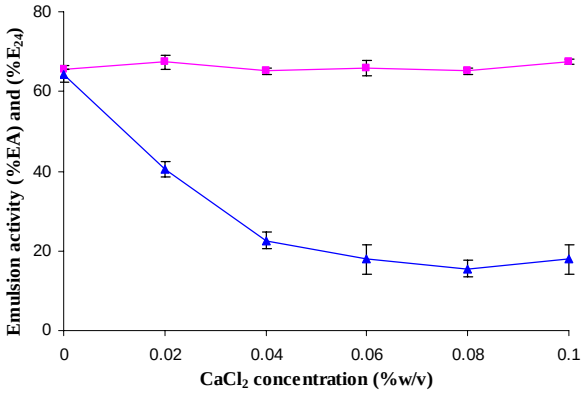


Figure 17. Effects of (a) NaCl (b) $MgCl_2$ (c) $CaCl_2$ concentration on emulsion activity of the yeast bioemulsifier ■: emulsification activity (% EA), ▲: emulsification index (% E_{24}). Bars represent the standard deviation from triplicate.

3.4.4 Determination of droplet size distribution

One common way to characterize the size distribution of the emulsions is the use of parameters that indicate the dispersion. The droplet size distribution influences the properties of the emulsions in such aspects as a long-term stability (Mcclements, 1999). The mean diameters of the droplets of d(10), d(50), d(90) and Span are summarized in Table 16. It is generally important that emulsion droplets are made as small as possible. A convenient way to evaluate the relative effectiveness of an emulsifier is to determine droplet size distribution. The droplet size of bioemulsifier was compared with commercial emulsifiers (gum arabic and lecithin). There were 3 pieces of information on distribution. The d(10) showed that there are about 10% of smaller droplets (μm) in the distribution. The d(50) indicated that half of all droplets (μm) are there in the distribution. The d(90) showed that about 90% of smaller droplets (μm) are there in the distribution. In addition, the span indicates the width of the distribution regardless of the median size (Palozolo *et al*., 2004). Vegetable oil, which gave the highest emulsion of each emulsifier, was used for the investigation. The median diameter of the emulsions was expressed ($d_{v,0.5}$). All the emulsions showed a monomodal distribution of droplets. Emulsion from the yeast bioemulsifier had a $d_{v,0.5}$ with 50% of the particles under 123.39 μm, compared with emulsion from gum arabic and lecithin which had 105.67 μm and 86.46 μm, respectively. This revealed that the emulsion from lecithin had the smallest particle size. However, the span of lecithin emulsion was not the lowest. It showed that emulsion from lecithin had the broadest range of polydispersity of the emulsion than did the others. Particle size distribution is a key characteristic, as it contributes to the physical stability property of the emulsion. The stability of an emulsion can be enhanced by reducing the droplet size. As a rule, large globules tend to coalesce faster than the small ones (Mcclements, 1999). Therefore, obtaining an emulsion with a uniform smaller droplet size becomes essential to achieve a stable emulsion system.

Table 16. Droplet mean diameter and dispersity index (span) of emulsions.

Samples	d(10)* µm	d(50)* µm	d(90)* µm	Span
Yeast bioemulsifier	40.49	123.39	241.58	1.63
Gum arabic	35.68	105.67	189.05	1.45
Lecithin	26.45	86.46	170.75	1.67

* The experiments were done in triplicate and results were reported as the average from triplicate determinations.

3.4.5 Preparation of salad dressing

Finally, the yeast bioemulsifier was tested in a salad dressing by using four different formulations. The results revealed that salad dressing made by adding three kinds of emulsifier (the yeast bioemulsifier, gum arabic and lecithin) had appropriate emulsion stability after leaving it at 4ºC for 1 h. On the other hand, the control formulation with no added emulsifier, showed rapid separation of oil. Every formulation showed emulsion activity higher than that of the control formulation and the difference was significant ($p>0.05$). The highest emulsification activity was found in the formulation with 0.6% (w/v) of the yeast bioemulsifier added (41%) and with 0.6% (w/v) gum arabic added (39%) respectively. Moreover, the results relating to the droplet size of emulsions are shown in Figure 18. The results indicate that the droplet sizes of emulsions with added emulsifier were smaller than the control and the difference was significant ($p<0.05$). Droplet size distribution of every formulation was in the range of 119.52-315.24 µm. The control formulation showed the highest droplet size of emulsion whereas adding 0.4% (w/v) of the yeast bioemulsifier and 0.4% and 0.6% (w/v) of gum arabic gave the smallest droplet size. The difference was not significant ($p>0.05$). Consequently, it could be feasible to use the yeast bioemulsifier in food products as a commercial emulsifier.

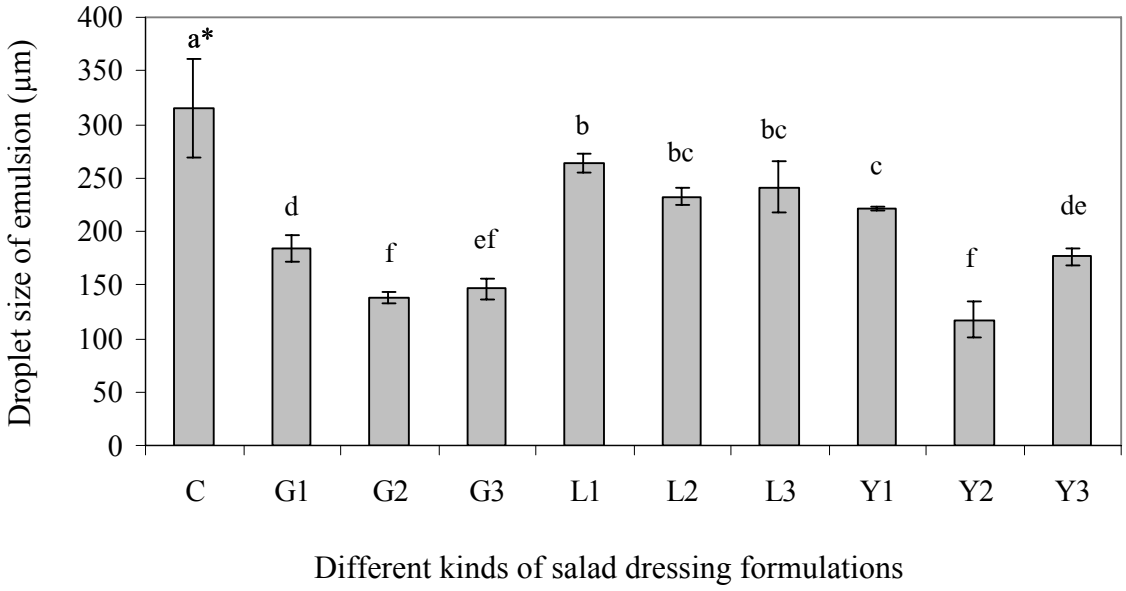


Figure 18. Droplet sizes of emulsion of salad dressing formulations at different concentration of emulsifier. (C : control (without emulsifier), G1 : gum arabic 0.2%, G2 : gum arabic 0.4%, G3 : gum arabic 0.6%, L1 : lecithin 0.2%, L2 : lecithin 0.4%, L3 : lecithin 0.6%, Y1 : yeast bioemulsifier 0.2%, Y2 : yeast bioemulsifier 0.4%, Y3 : yeast bioemulsifier 0.6%). * Different letters indicate significant differences ($p<0.05$).

3.5 Conclusion

The bioemulsifier from yeast, *Saccharomyces cerevisiae*, isolated from palm sugar wine obtained from distillation of local brewers could be a source of raw material for the mass production of bioemulsifier. This would not only eliminate the need to grow the yeast specifically for the production of emulsifiers, it helps the environment by reducing waste discharge from local distillers. These findings reveal that the product obtained could be very useful in situations which extreme conditions of temperature, salinity and pH 5-8 are present. The capacity to form emulsion with vegetable oils suggests a potential application as an emulsifying agent in the food industry such as in salad dressing. In addition, the production of the yeast bioemulsifier would be economically favorable, since the process converts a low-value waste into a high-value product.

CHAPPER 4

CHARACTERIZATION OF AN UNEXPECTED BIOEMULSIFIER FROM SPENT YEAST OBTAINED FROM THAI TRADITIONAL LIQUOR DISTILLATION

4.1 Abstract

Crude biopolymer was extracted from spent yeast, lyophilized and fractionated on Sephadex G-100 to yield two fractions coded as fraction I and II. Fraction I was composed of both carbohydrates and proteins, showing emulsifying activity whereas fraction II consisted of only proteins and possessed no activity. Hence composition and chemical characterization of the purified fraction I (bioemulsifier) was analyzed using various analytical techniques. It was found that the sample contained 96% of carbohydrates consisting mainly of glucose with minor quantities of mannose, and 4% of protein built from 17 amino acids with the highest content of serine followed by alanine. The results also indicated that the sample was protein-bound glucan with the average molecular weight of 1.93×10^5 Da. The functional groups and primary structure of the sample were revealed by FTIR and NMR techniques. The data demonstrated that the sample comprises a mixture of (1→4)-α- and (1→3)-β-D-glucans bound with protein. Enzymatic hydrolyses using α-amylase and β-1,3-glucanase confirmed the presence of both glucans. Therefore, this spent yeast bioemulsifier was identified as glucan-protein complex which is different from usual mannoprotein emulsifier derived from yeasts.

4.2 Introduction

Cell wall of *Saccharomyces cerevisiae* is organized as two layers that consist of three main groups of polysaccharides: polymers of mannose covalently linked to peptides (mannoproteins, 40% of the cell wall dry mass), polymer of glucose (β-glucan, 60% of the cell wall dry mass), and polymer of *N*-acetylglucosamine (chitin, 1-3% of the cell wall dry mass) (Lipke and Ovalle, 1998). These components are all interconnected by covalent bonds (Kapteyn *et al*., 1999). Outer layer consists of mannoproteins composed of a minor part of proteins which is covalently linked to the major part of a mannan polymer (Vinogradov *et al*., 1998). One of the mannoproteins is a long branched polymer made of (1→6)-linked-α-D-mannopyranosyl residues with numerous (1→2)-linked-α- and (1→3)-linked-α- side chains. These longer polysaccharides are attached to protein by *N*-linkages via asparagine residues. Another type is a short mannan consisting of only 1 to 5 mannose units attached to protein by *O*-linkages via serine or threonine (Klis, 1994). Inner layer consists of β-D-glucan chitin (Lipke and Ovalle, 1998; Feuillat, 2002). The β-D-glucan occurs as short chains with approximately 150 units of (1→6)-β-D-glucopyranose attached to long backbone chains of (1→3)-β-D-glucopyranose with about 1500 residues (Lipke and Ovalle, 1998; Feuillat, 2002). Recently, β-D-glucan and mannoprotein that are components of yeast cell wall have received much more considerable attention. β-glucan plays an important role in an immune system that protects the body against viral, bacterial, and fungal infections (Yadomae and Ohno, 1996; Lee *et al*., 2001; Kim and Yun, 2006). Moreover, β-D-glucan has been widely used in the formulation of food systems such as a fat replacer in mayonnaise (Thammakiti *et al*., 2004; Worrasinchai *et al*., 2006). Whereas mannoprotein has been shown to be an effective bioemulsifier (Cameron *et al*., 1988; Torabizadeh *et al*., 1996; Barriga *et al*., 1999).

It is well known that yeast (*S. cerevisiae*) has been used in baking and fermenting alcoholic beverages for thousand years. Spent yeast becomes a major by-product, especially from wine or brewer's industry, which is only used as a protein supplement in animal feed. It is still an underutilized resource and its disposal is often an environmental problem. Apart from wine or brewer's industry, another source of

S. cerevisiae biomass is from a traditional liquor distillation. The traditional distilled liquor produced in Southeast Asia are broadly classified into (1) rice and cereal wine, (2) palm wine, and (3) distilled spirit from rice, cereal, or palm wine. Nowadays, the distilled liquor from palm wine is more popular traditional distilled liquor than its origin (palm wine). Unique and traditional distillation method is still used in this area. Basically, the common method for making the Thai traditional distilled liquor from palm wine is as follows: sap of palm tree (*Borassus flabillifer* Linn.) is concentrated to palm syrup by heating and then fermented by back-slopping technique. The obtained palm wine is distilled for 2-3 hours without separation of yeast cells. After distillation huge amount of waste containing yeast cells is discarded. Therefore, the high-value-added products from this resource are great interesting. In addition, it will be help to minimize an environmental impact from the manufacturers. In this study, an unexpected bioemulsifier was isolated from the spent yeast obtained from Thai local palm wine distillation. This spent yeast bioemulsifier will be hereafter described and characterized.

4.3 Materials and Methods

4.3.1 Extraction and purification of the spent yeast bioemulsifier

4.3.1.1 Extraction method

As shown in Figure 19, distillate residues obtained from a local distillery in Songkhla Province, Thailand were centrifuged at 6,000×*g* for 10 min at 4ºC. Yeast cell pellets were washed twice in a normal saline. Twenty percent (w/v) of yeast cells were suspended in distilled water containing 0.1 M potassium citrate and 0.02 M potassium metabisulfite. pH of the suspension was then adjusted to pH 7.0 with 1 M NaOH. After that the cell suspension was autoclaved (121ºC) for 30 min (Barriga *et al*., 1999). The resulting suspension was centrifuged at 6,000×*g* for 10 min at 4ºC. The supernatant was retained and mixed with five volume of chilled ethanol, incubated overnight at 4ºC for complete precipitation. The sample was centrifuged at 6,000×*g* for 10 min at 4ºC. After centrifugation, the supernatant was discarded and the precipitate was washed twice with chilled ethanol. Then, the precipitate was dialyzed

against distilled water (molecular weight cut off 8000 Da) for overnight and lyophilized prior to purification.

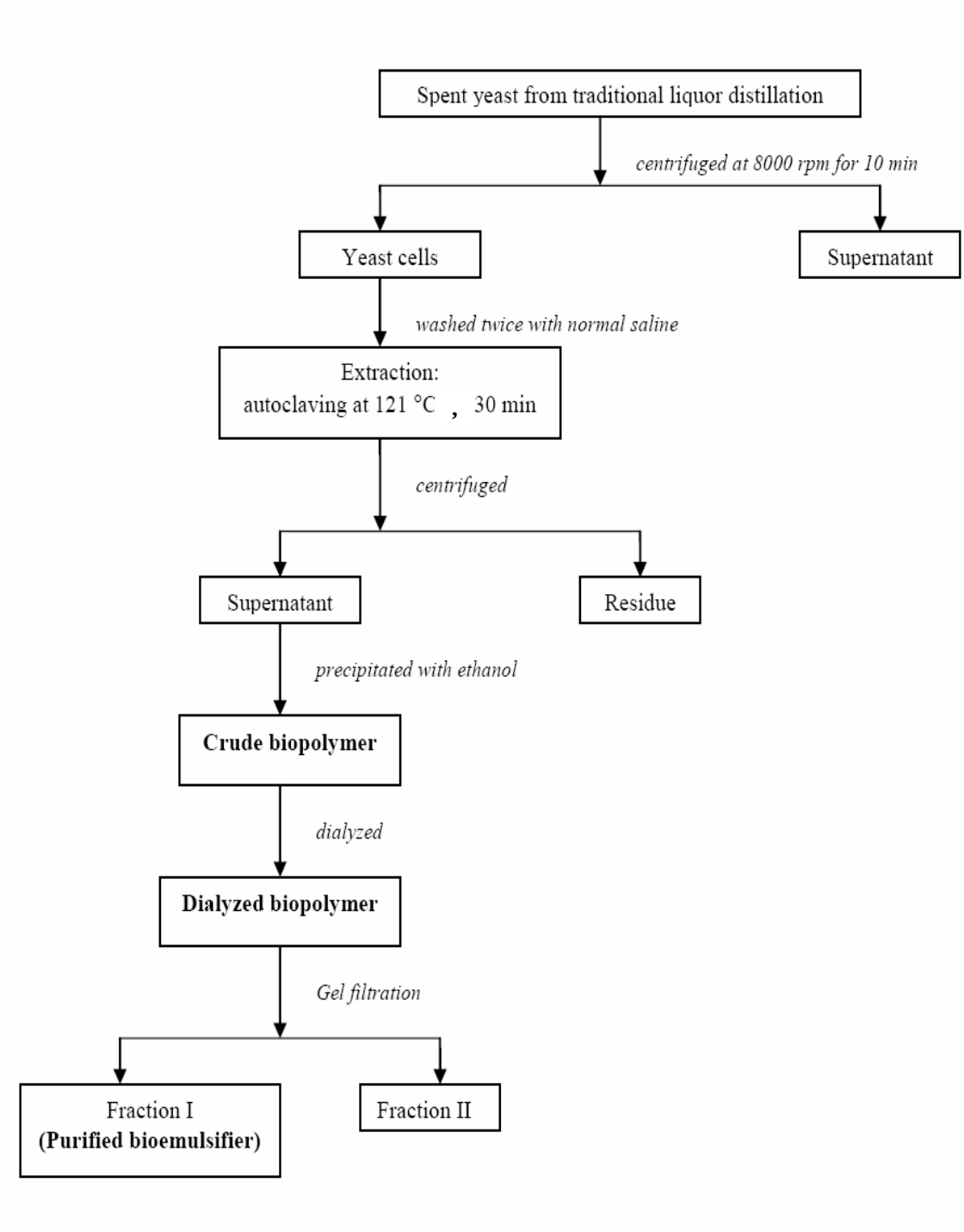


Figure 19. Scheme for the preparation of bioemulsifier from spent yeast.

4.3.1.2 Gel-filtration chromatography

Dialyzed biopolymer (10g) was dissolved in 50 ml distilled water. The sample was further purified by gel filtration chromatography on Sephadex G-100 (Pharmacia, Sweden) column (2.6×30 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.3) containing 150 mM NaCl at 0.4 ml/min. Samples obtained from all steps were subjected to the measurement of protein content (Bradford, 1976), total sugar (Dubois *et al*., 1956) and uronic acid content by carbazole assay (Chaplin and Kennedy, 1986).

4.3.2 Emulsification property

Crude and purified samples were dialyzed against water, freeze dried, and then determined an emulsification activity according to a method of Cameron *et al*. (1988) with a slight modification. One milliliter of vegetable oil (soybean oil, palm oil, corn oil, olive oil, sunflower oil, rice bran oil or sesame oil) was added to 1 ml of the sample suspension (20 mg/ml) and vortexed at high speed for 3 min. The mixture was allowed to stand for 24 h (emulsification index, $\%E_{24}$) prior to measurement. The emulsion activity is defined as a height of an emulsion layer divided by total height and expressed as percentage. The experiments were carried out in triplicate and the results were reported as an average value. In addition, type of emulsion formed was investigated by using filter paper wetting test and dilution test (Lieberman *et al*., 1988). Statistical analysis was performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). Data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan's multiple-range test.

4.3.3 Determination of the spent yeast bioemulsifier composition

4.3.3.1 Monosaccharides composition

Monosaccharide composition of the purified spent yeast bioemulsifier was investigated according to a method reported by Methacanon *et al*. (2005). Briefly, sample (~ 25 mg) was soaked in trifluoroacetic acid (conc. TFA, 2 ml) for overnight at ambient temperature. Then, TFA was diluted to 3 M and the sample was hydrolyzed at 120°C for 5 h. The TFA was removed using a rotary vacuum evaporator. Distilled water was subsequently added to solid in order to wash the sample and followed by re-

evaporation; the procedure was repeated until the obtained hydrolysate was neutral. Finally, dry hydrolysate solid was dissolved in water for monosaccharide analysis by HPLC-ELSD. Twenty microliters of the hydrolysate were applied to a Prevail Carbohydrate ES (250×4.6 m). Products monitored by RI (refractive index) detector were eluted by 78% (v/v) of acetonitrile with a flow rate of 1 ml/min at room temperature. Mannose and glucose were used as monosaccharide standards.

4.3.3.2 Total amino acid composition

Purified spent yeast bioemulsifier (~0.1 g) was hydrolyzed with 5 ml of 6 N HCl for 16 h at 110ºC. After complete hydrolysis, the volume was adjusted with nanopure distilled water to a final volume of 25 ml. The amino acids were determined by RP-HPLC according to the Waters AccQ.Tag method (Millipore Co-Operative, Milford, MA, USA) as described by Hughes *et al*. (2002). This method involved pre-column derivatization and conversion of the amino acids to stable fluorescent derivatives by reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. Separation and analysis of amino acid derivatives was performed using an Alliance® HPLC System (Waters) equipped with an AccQ.Tag C18 column (3.9×150 mm) at 37ºC. AccQ.Tag eluent, acetonitrile and distilled water were used as mobile phase. After that, the eluent was monitored at an excitation wavelength 250 nm and emission at 395 nm. The peak area of each amino acid was processed by the Empower software (Waters) and the concentration of amino acid was calculated by compared with the amino acid standard and expressed as mol%.

4.3.3.3 Elemental analysis (CHNS)

Total carbon, hydrogen, nitrogen and sulphur contents of the purified spent yeast bioemulsifier were determined using the Thermo Quest FlashEA™ 1112 CHNS analyzer (Italy). The Dynamic Flash Combustion was used in this technique.

4.3.4 Chemical characterization

4.3.4.1 Determination of molecular weight

An average molecular weight of the purified spent yeast bioemulsifier was determined by a gel permeation chromatography (GPC). The analysis was performed on Ultrahydrogel linear column (7.8 i.d. × 300 mm, Water, USA) connected in line with a Water GPC apparatus. The universal calibration was obtained

by using pullulan standards with molecular weight ranging from 5,900 to 788,000 Dalton. The purified spent yeast bioemulsifier (2 mg/ml) was eluted with 0.05 M sodium bicarbonate buffer pH 11 with a flow rate of 0.6 ml/min at 25°C and monitored by RI and photodiode array detectors.

4.3.4.2 FT-IR

Structural characteristics of the crude and purified spent yeast bioemulsifier were determined using Fourier Transform IR spectrophotometer (Bruker, Equinox 55, Germany). The purified spent yeast bioemulsifier was ground, mixed with KBr powder and then pressed to form a pellet for measurement in a frequency range of 4000-400 cm^{-1}.

4.3.4.3 ^{1}H and ^{13}C NMR

NMR data was obtained to elucidate the purified spent yeast bioemulsifier chemical structures. The sample was recorded as solution in D_2O. ^{1}H and ^{13}C NMR spectra were recorded on a Model Varian Unity® Inova 500 spectrometer (Germany) (operating frequency of 499.573 MHz for ^{1}H and 125.632 MHz for ^{13}C). The chemical shifts were expressed in ppm relative to a resonance of Me_4Si (an internal standard).

4.3.5 Enzymatic hydrolysis

The purified spent yeast bioemulsifier was dissolved in water (10 mg/ml) and diluted in 20 mM acetate buffer pH 5.0 to obtain the final concentration of 2 mg/ml. Then, the sample was hydrolyzed with β-1,3-glucanase (EC 3.2.1.39) from *Helix pomatia* 1.5 units/mg (Sigma-Aldrich, Steinheim, Germany) by incubating at 37°C for 4 h. After that the reaction was stopped by boiling for 10 min. The extent of hydrolysis was estimated from the amount of reducing sugars. The reducing sugars were determined as D-glucose equivalents using the dinitrosalicylic acid (DNS) assay (Miller, 1959). The resulting solution was also analyzed by HPLC. Twenty microliters of the hydrolysate were applied to a Zorbax-NH_2 (4.6 × 250 mm). Products were eluted by 75% (v/v) of acetonitrile with a flow rate of 0.7 ml/min at room temperature and monitored by RI (refractive index) detector. Glucose was used as monosaccharide standard. For α-amylase hydrolysis, the dissolved sample (10 mg/ml) was diluted in 50 mM phosphate buffer pH 7.0 to obtain the final concentration of 2 mg/ml. Then, the

sample was hydrolyzed with α-amylase (EC 3.2.1.1) from *Bacillus species* Type XI-A 90 units/mg (Sigma-Aldrich, Steinheim, Germany) by incubating at 20°C for 1 h. After that the same manners were performed as mentioned above.

4.4 Results and Discussion

4.4.1 Extraction and purification of the spent yeast bioemulsifier

Crude extracted biopolymer was 0.53 g/g of dry weight yeast biomass. As shown in Table 17, it was composed of proteins (9%) and carbohydrates (91%). After dialysis, it was found that the proteins content decreased approximately 2% due to removal of low molecular weight free proteins. The dialyzed sample was further purified using Sephadex G-100. Figure 20 shows the gel filtration chromatograms of the dialyzed sample eluted by Tris-HCl buffer containing NaCl. Two fractions separated from the column, named as fraction I and II, were shown. The fraction I consisted of both carbohydrates and proteins whereas the fraction II had only proteins. In addition, it was found that the fraction I could stabilize emulsion toward palm oil with the emulsification index ($\%E_{24}$) approximately 63% (Table 17), on the other hand, the fraction II possessed no emulsion activity. Therefore, the fraction I (purified bioemulsifier) was considered of interest in further studies. It was found that the purified bioemulsifier from yeast cell wall consisted of 4% proteins and 96% carbohydrates. The results suggested that proteins were probably bonded with carbohydrates to form a complex.

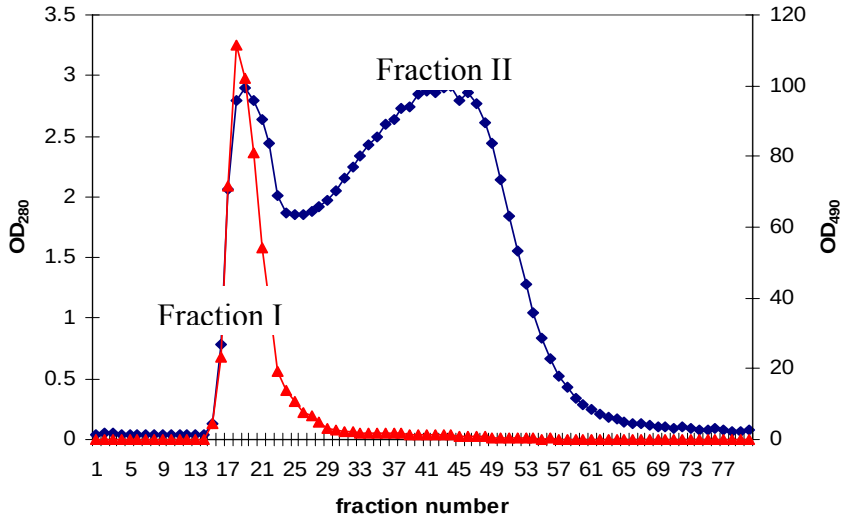


Figure 20. Fractionation of the biopolymer using gel filtration on Sephadex G-100 column that was eluted with 10 mM Tris-HCl buffer (pH 7.3) containing 150 mM NaCl at 0.4 ml/min. The presence of proteins in eluent was detected by measuring optical density at 280 nm (♦ OD_{280}), and that of sugars by measuring optical density at 490 nm (▲OD_{490}).

Table 17. Composition and emulsification index (%E_{24}) activity of biopolymers obtained from each preparation step.

Samples	**Total protein[a] (% w/w)**	**Total sugar[b] (% w/w)**	**Emulsification index (%E_{24})**	**Yield[c] (%)**
Crude biopolymer	9.2	90.8	62.06	100.0
Dialyzed biopolymer	6.6	93.4	61.54	27.6
Purified bioemulsifier				
(fraction I)	3.8	96.2	62.96	17.7
(fraction II)	2.8	ND	ND	ND

[a] measured by Bradford method with bovine serum albumin as a standard.

[b] measured by phenol-sulfuric acid method with glucose as a standard.

[c] based on dry weight of crude biopolymer after precipitation.

ND: not determined.

4.4.2 Emulsification properties of the spent yeast bioemulsifier

Specificity of the spent yeast bioemulsifier to various vegetable oils was investigated. The results showed that the bioemulsifier could form stable emulsion with all tested vegetable oils (Fig. 21). The highest emulsifying activity was observed with olive oil, corn oil, sunflower oil and palm oil, which were not significantly different ($p>0.05$). It had emulsifying properties as good as those of the commonly used food emulsifiers, gum arabic and lecithin (data not shown). In addition, the bioemulsifier promoted formation of oil-in-water emulsion since it was found that emulsions dispersed rapidly on filter paper and dispersed cloudy in water, and remained a droplet in oil. The presence of hydrophilic glucose polymers bound proteins provided a structure of this spent yeast bioemulsifier with emulsification property. This is clearly that polymers are not efficient in reducing interfacial tension but form excellent interfacial barriers. Polymers act to prevent coalescence and are useful as emulsifying agent (Dickinson and McClements, 1996). The result was agreement with another known high molecular weight bioemulsifier that shown to be an effective bioemulsifier. For instance, a bioemulsifier from *Curvularia lunata* IM2901 contained a complex protein (25%) and polysaccharide (48%) which sugar component was identified as a polymer of D-glucose (Paraszkiewicz *et al*., 2002). Rosenberg *et al*. (1979) reported on emulsan, heteropolysaccharide-protein bioemulsifier from *Arthrobacter* RAG-1. Liposan, an extracellular emulsifier synthesized by *Candida lipolytica*, was composed of approximately 83% carbohydrates and 17% proteins. Liposan stabilized oil-in-water emulsion with a variety of commercial vegetable oils. The greatest stabilizing effect by liposan was for the cotton seed, corn, soybean and peanut oil emulsions (Ciligliano and Carman, 1985). In addition, Phetrong *et al*. (2008) reported on bioemulsifier from *Acinetobacter calcoaceticus* SM7. Preliminary chemical characterization of partially purified bioemulsifier composed of 57.74% carbohydrate and 42.26% protein. Bioemulsifier extracted from *A. calcoaceticus* SM7 was capable of emulsifying both pure aliphatic and aromatic hydrocarbons.

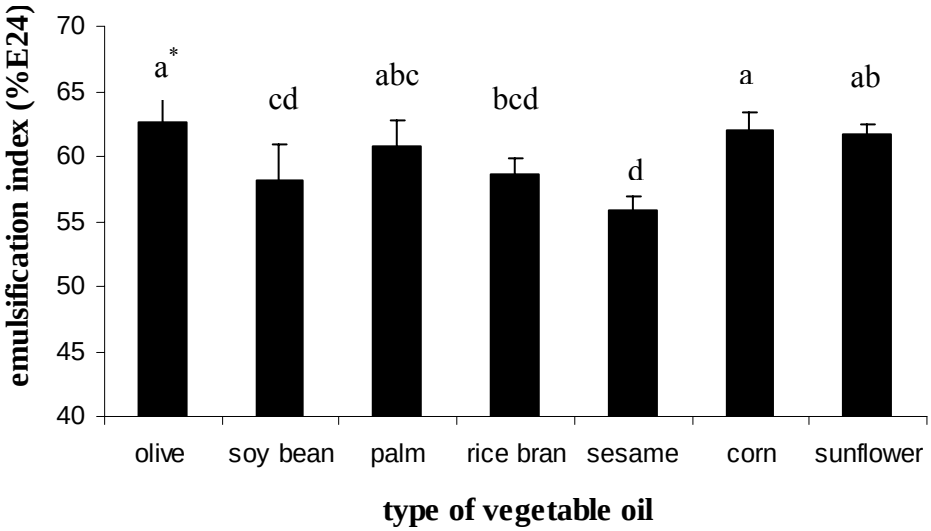


Figure 21. Emulsification index (%E_{24}) of the spent yeast bioemulsifier with various vegetable oils.

* Different letters above bar indicate significant differences ($p>0.05$).

4.4.3 Determination of the spent yeast bioemulsifier composition

Monosaccharide composition of the purified spent yeast bioemulsifier was analyzed by HPLC-ELSD. It was found that glucose was a major sugar accounted for 95% with a small proportion of mannose approximately 5%, indicating the presence of glucan (Fig. 22). This result was surprising since an extraction with heat at neutral extraction condition was expected to solubilize the structural mannoprotein in the outer layer of the yeast cell wall, according to previous work (Cameron *et al.*, 1988). This discrepancy might be due to the difference in a liquor distillation process. As mentioned earlier, in Thai traditional liquor distillation, palm wine is directly boiled without separation of yeast cells for 2-3 h until distilled liquor is obtained. Thereby, mannoprotein which is water soluble could be leached away from the cell walls during the distillation process, resulting in less amount of mannoprotein left in the studied spent yeast. In addition to the presence of glucan in the purified bioemulsifier, protein was detected using Bradford protein assay (Table 1). Then, amino acids was determined and demonstrated as mol% in Table 18. Serine was found in the highest content (15 mol%), followed by alanine (13 mol%) and threonine

(11 mol%), respectively. Acidic amino acids like glutamine acid and aspartic acid were also detected (ca. 9-10 mol%).

The CHNS elemental contents were reported on weight percent (Table 19). Elementary analysis of the purified spent yeast bioemulsifier showed the presence of carbon (33% C), hydrogen (6% H) and nitrogen (0.5% N). No significant quantity (<0.01%) of sulphur was found, which corresponded well with the amino acid result showing a significantly low content of cysteine.

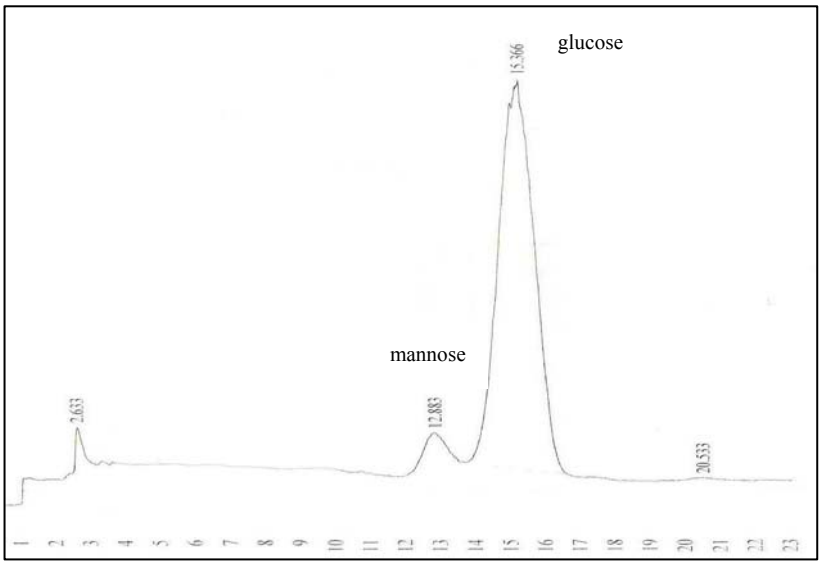


Figure 22. HPLC chromatogram of the purified spent yeast bioemulsifier; mannose was eluted at 12.885 min and glucose was eluted at 15.366 min.

Table 18. Amino analysis of the purified spent yeast bioemulsifier.

Amino acid	Mol composition (%)
Asp	8.88
Ser	14.86
Glu	9.84
Gly	7.31
His	0.89
Arg	1.47
Thr	11.06
Ala	12.67
Pro	7.58
Cys	0.51
Tyr	2.19
Val	6.56
Met	0.85
Lys	3.38
Iso	3.82
Leu	5.50
Phe	2.63

* Tryptophan was not detected in this method.

Table 19. Chemical properties of the purified spent yeast bioemulsifier.

Analysis	Content (%)
Elementary composition	
C	33.01
H	5.59
N	0.52
S	less than 0.01
Presence of	
Protein	3.83
Carbohydrate	96.17

4.4.4 Chemical characterization

The average molecular weight of the purified spent yeast bioemulsifier was estimated by Gel Permeation Chromatography (GPC) with apparent average molecular weight (M_w) approximately 1.93×10^5 dalton (Fig. 23). For identification of the main functional groups present in the purified bioemulsifier, it was submitted to FT-IR and NMR analyses. Figure 24 shows the FT-IR spectra of the crude and purified spent yeast bioemulsifiers. The main absorptions are characteristic of polysaccharides, related to O-H stretching (3408 cm^{-1}) and a weak C-H stretching (2931 cm^{-1}). Characteristic bands at 1652 and 1416 cm^{-1} are corresponded to asymmetry and symmetry stretching vibration of anion carboxylate (COO^-), respectively (Gonzaga *et al*., 2005). It is worth noting that both peaks were greatly reduced and slightly shifted when compared with those in crude sample due to removal of other impurities including some proteins. In the mean time, the C-O stretching bands observed in the range of 1200-950 cm^{-1} which generally known to be a typical characteristic of the polysaccharide region become stronger in the purified sample. The coupling modes of C-O and C-C stretching of α- and β-glucans were observed at 1154 and 1080 cm^{-1}, respectively. Band at 1023 cm^{-1} could be originated from α-glucan (Sandula *et al.,* 1999). Band at 922 cm^{-1} was attributed to the glycosidic linkages. In addition, there is an anomeric region at 950-750 cm^{-1}, which possible to distinguish band characteristic for α or β configuration (Rout *et al*., 2005). For

glucans, bands at approximately 850 and 890 cm^{-1} are assigned to the α- and β-glucosidic linkages, respectively (Sandula *et al.,* 1999). In the purified bioemulsifier, there were characteristic absorption bands at 851 cm^{-1}, demonstrating α-D-glucosidic linkage. The FT-IR spectra also exhibited complex vibrational modes at low wavenumbers (below 800 cm^{-1}) due to the skeletal mode vibration of the glucose pyranose ring. Due to low protein content, N-H vibration generally expected at 3400 cm^{-1} could be overlapped by O-H stretching vibration as well as other protein characteristic bands generally observed at 1600-1700 cm^{-1} (amide I) and 1510-1580 cm^{-1} (amide II).

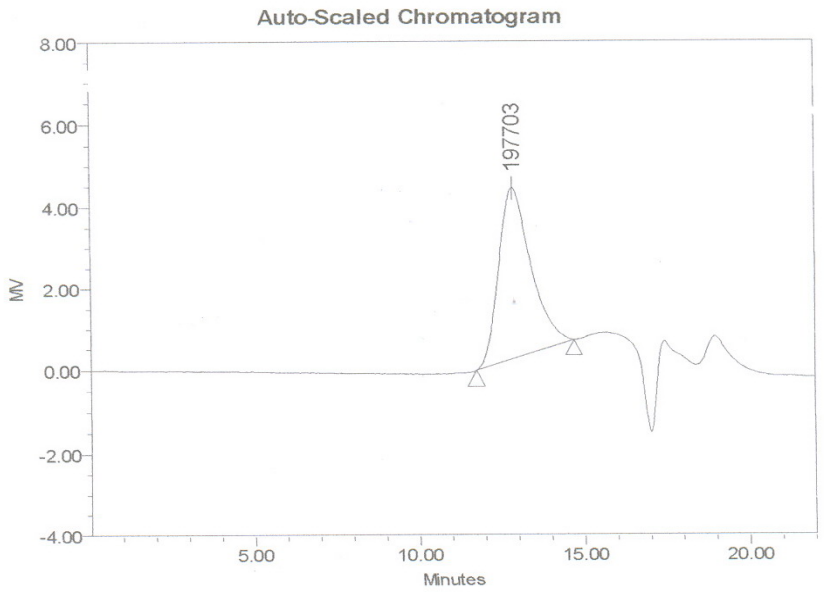


Figure 23. Molecular weight distribution of the purified spent yeast bioemulsifier by gel permeation chromatography.

Figure 25 shows ^{1}H-NMR spectrum of the purified spent yeast bioemulsifier dissolved in D_2O. As can be seen, proton signals appear as complex series ranging from 3.2 to 5.4 ppm. From the literature, chemical shifts of an anomeric region were observed in range from 4.1 to 4.5 ppm, corresponding to the β configuration and from 4.9 to 5.6 ppm, corresponding to α configuration (Gonzaga *et al*., 2005; Chandra *et al*., 2007). On the basis of the integrated proportions of both regions, the quantitative estimation of the β- and α-glucans ratio in the purified spent

yeast bioemulsifier was calculated as approximately 0.27:1. The chemical shifts from 3.2 to 4.0 ppm were assigned to protons of carbons C2 to C6 of sugar ring, which often becomes crowded by the overlap of signals (Gonzaga *et al*., 2005; Ghosh *et al.,* 2008). The protein groups related to the glucan-protein structure likely seems to be present. The chemical shifts detected at 1.7 ppm attributed to N-CH_3 and N-H which are generally observed at 0.5 to 3.0 ppm (Gonzaga *et al*., 2005).

^{13}C-NMR spectrum of the purified spent yeast bioemulsifier dissolved in D_2O is shown in Figure 26. The complex nature of the spectrum strongly suggests that the sample was a mixture which is consistent with a variety components found in yeast cell wall. The NMR result revealed evidence of the α- and β-configurations of the anomeric carbon (C1) with signals close to 100.5 and 102.9 ppm, respectively (Chandra *et al*., 2007). This is in accordance with the result obtained by FT-IR, confirming the presence of α- and β-glucans in the purified spent yeast bioemulsifier. Furthermore, the signal at 100.5 ppm was higher than that at 102.9 ppm, suggesting predominantly α-glucan. Interpretation of the ^{13}C-NMR spectrum of the purified spent yeast bioemulsifier (Table 20) was based on a set of chemical shifts from the literatures (Chandra *et al*., 2007; Rout *et al*., 2005; Tada *et al.*, 2009). It is also worth noting that (1→3)-β-D-glucan generally shows a characteristic chemical shift of C3 at around 85 ppm (Tada *et al.*, 2009). However, the C3 signal of this sample was absent, probably due to thermal denaturation and degradation of the glucan during distillation and/or autoclaving steps (Adachi *et al*., 1990). Adachi *et al.* (1990) showed that long term heating gradually degraded the (1→3)-β-D-glucan. Moreover, thermal denaturation of (1→3)-β-D-glucan in neutral aqueous solution occurred above 130°C, resulting in polymer conformation change and consequently influencing on its physicochemical properties. The ^{13}C-NMR of a carbonyl carbon is in the range of 160 to 220 ppm, depending on surrounding atoms. The resonances between 160 and 185 ppm are generally assigned to carboxylic acids (-COOH) mainly organic acid that are free or involved in esters or amides (Breitmaier and Voelter, 1987). In the present case, two signals at 180.6 ppm and 178.7 ppm were observed in this region. The first one was probably generated by the carboxylate of uronic acid, which is consistent with the FT-IR result. The uronic acid was also detected in the sample by Carbazole assay using D-glucurono-6,3-lactone as a standard (data not shown). The last could be

attributed to the amide group (-CONH) of protein (Tao and Zhang, 2008) even though the additional signals between 20 to 50 ppm belonging to $–CH_3$ and $–CNH_3^+$. However, the presence of these groups was demonstrated in the ^{1}H-NMR result. Overall, the results indicated that the purified spent yeast bioemulsifier consisted of a mixture of α- and β-glucans bound with proteins. The α-glucan was presumed as glycogen which is one of the components found in yeast cell wall. Glycogen, a polymer of α-D-glucose joined by (1→4) and (1→6) linkages, is the energy reserve carbohydrate accumulated by *S. cerevisiae*, which can be mobilized during periods of yeast starvation. It can vary from as little as 1% to as much as 29% of the dry weight, depending upon the nutritional status of the cells, the method of isolation, the environmental conditions and the time the cells were harvested (Kwiatkowski *et al*., 2009). It has been reported that glycogen in *S. cerevisiae* is present in two pools, one soluble and intracellular, the other insoluble owing to its covalent linkage to cell wall β-glucan (Arvindekar and Patil, 2002). For β-glucan, it could be attributed to (1→3) (1→6)-β-D-glucan, which can be isolated from *S. cerevisiae* and has been studied extensively.

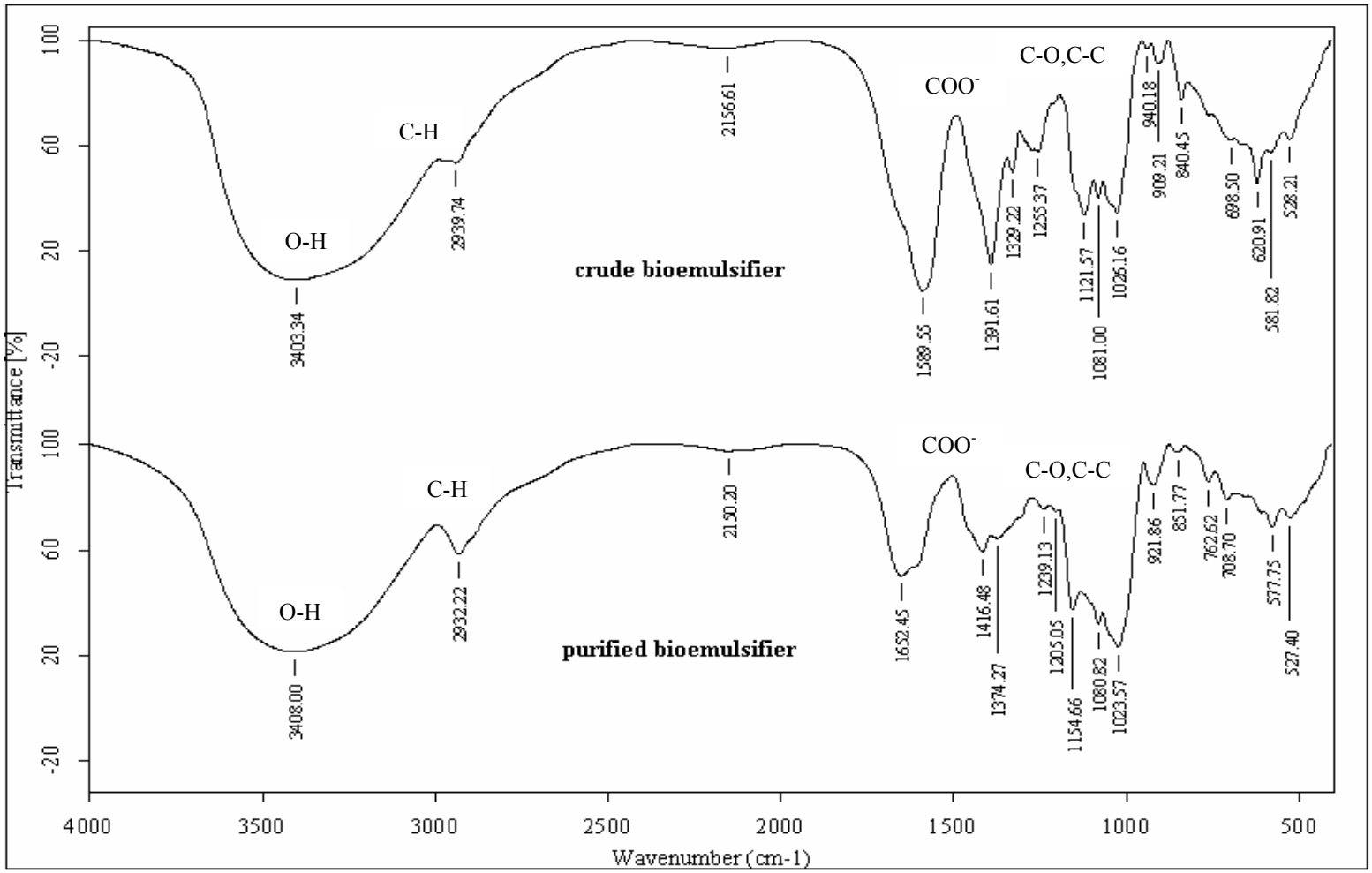


Figure 24. FT-IR spetra of the crude and purified spent yeast bioemulsifier.

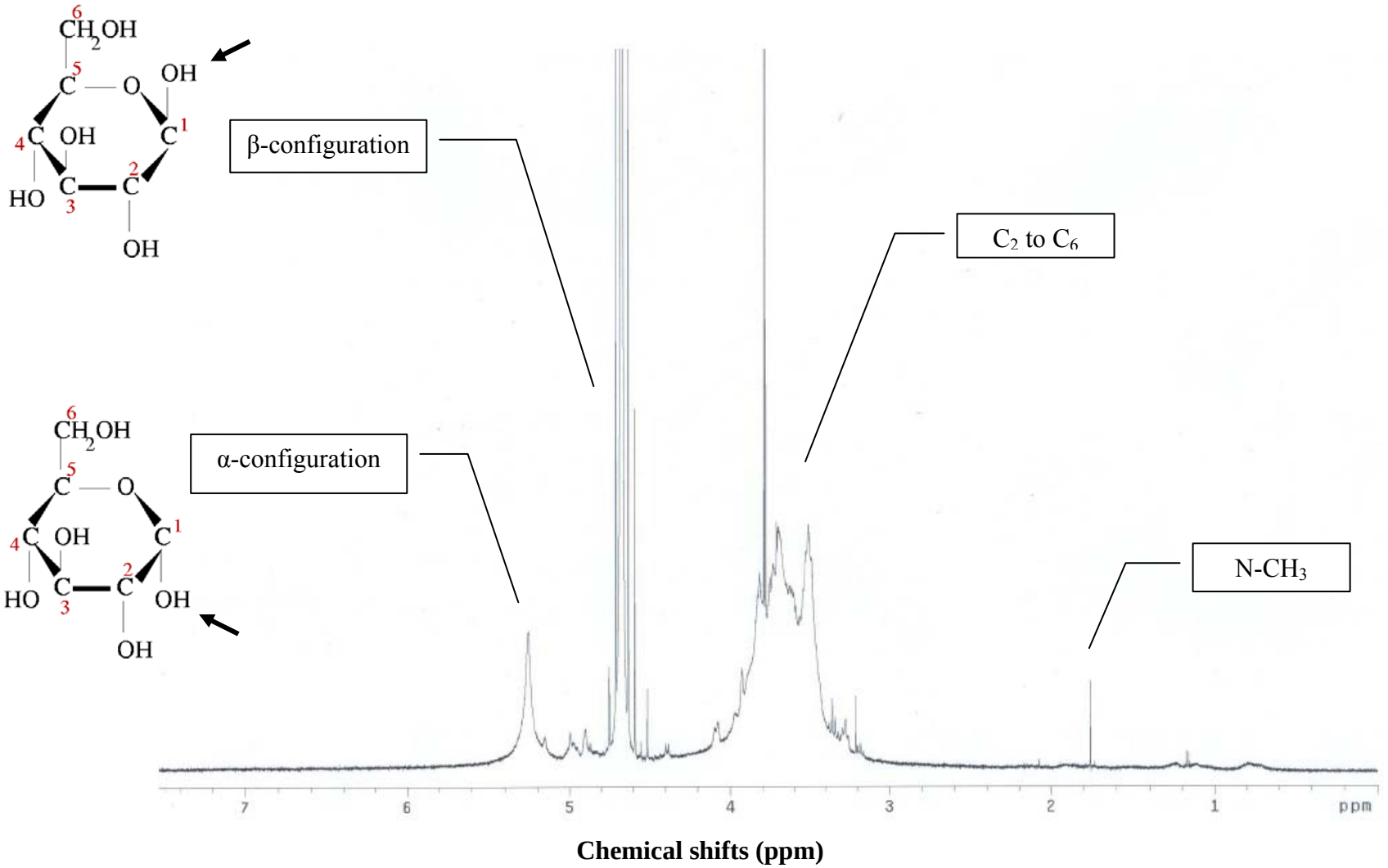


Figure 25. ^{1}H-NMR spectra of the purified spent yeast bioemulsifier (500 MHz, D_2O).

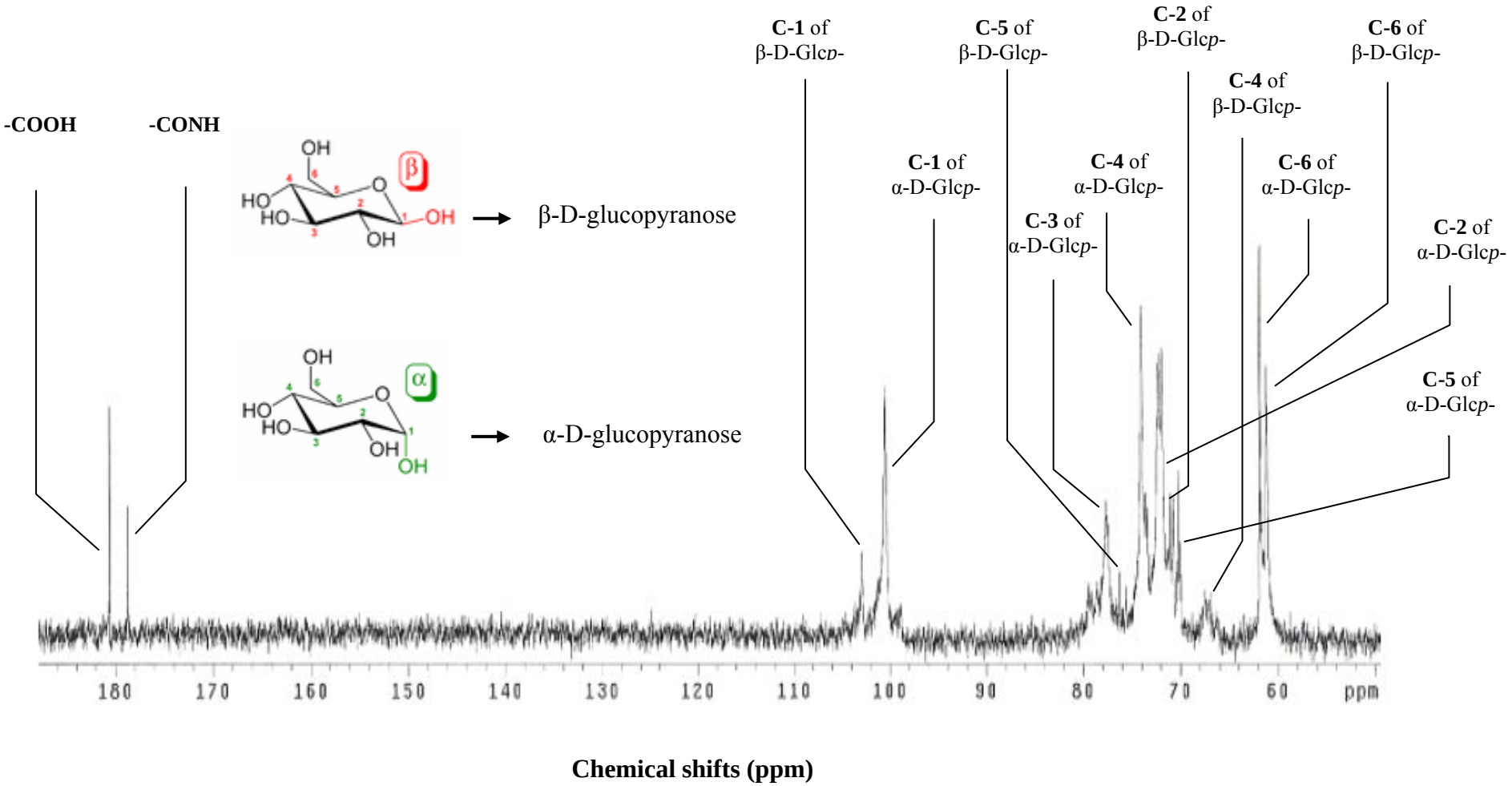


Figure 26. ^{13}C-NMR spectra of the purified spent yeast bioemulsifier (125 MHz, D_2O).

Table 20. ^{13}C-NMR assignments of the purified spent yeast bioemulsifier.

Glucan	C-1	C-2	C-3	C-4	C-5	C-6	Literature reference
Purified bioemulsifier							
→4)-α-D-Glc*p*-(1→	100.5	72.2	77.7	74.0	70.7	61.8	
→3)-β-D-Glc*p*-(1→	102.9	71.9	-	67.6	76.3	61.2	
→4)-α-D-Glc*p*-(1→	98.0	71.4	71.7	75.7	69.6	60.6	Chandra *et al.*
→3)-β-D-Glc*p*-(1→	102.4	72.3	85.1	68.9	75.9	60.7	(2007)
→3)-α-D-Glc*p*-(1→	100.1	70.0	80.4	69.3	72.5	61.1	Rout *et al.*
→3)-β-D-Glc*p*-(1→	103.1	73.0	85.2	68.8	76.4	67.0	(2005)
→3)-β-D-Glc*p*-(1→	103.0	73.1	86.1	68.6	76.4	61.0	Tada *et al.*
→3,6)-β-D-Glc*p*-(1→	103.0	72.9	85.8	68.7	75.0	68.6	(2009)

4.4.5 Enzymatic hydrolysis

Enzymatic hydrolysis is a specific for analysis of many polysaccharide structures. In the present study, β-(1→3)-glucanase (EC 3.2.1.39) and α-amylase (EC 3.2.1.1) were used for hydrolyses of (1→3)-β-D- and (1→4)-α-D-glycosidic linkages in the sample, respectively. The amounts of reducing sugar detected in the hydrolysates using glucanase and amylase were 55.0% and 38.5%, respectively. The data suggested that the β-glucan present in this sample possessed less branched chains than α-glucan since both enzymes only cleaves glycosidic linkages between two unsubstituted glucose residues. It was therefore confirmed that this spent yeast bioemulsifier comprised β-(1→3)-linked and α-(1→4)-linked D-glucans. The rest was non-hydrolyzed residues, possibly (1→6)-linked D-glucose branched chains and/or protein.

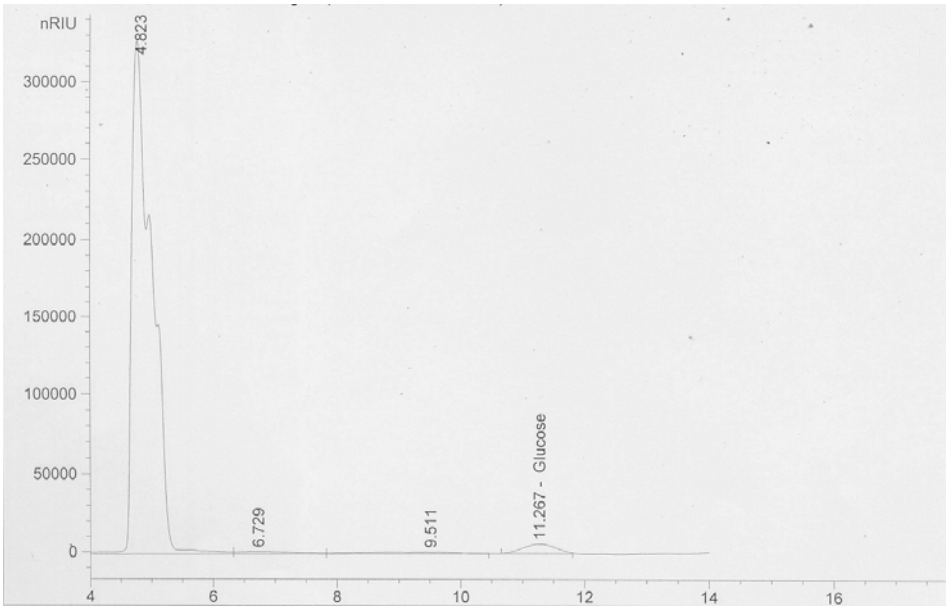


Figure 27. HPLC chromatogram of hydrolyzed spent yeast bioemulsifier, glucose was eluted at 11.267 min.

4.5 Conclusions

In contrast to previous published bioemulsifier from yeast cell wall which is declared as mannoprotein, in this study, the purified bioemulsifier from spent yeast obtained from Thai traditional liquor distillation was found as glucan-protein complex with average molecular weight (M_W) approximately 1.93×10^5 dalton. This spent yeast bioemulsifier predominantly consisted of α-(1→4)-D-glucan presumably as glycogen linked to β-(1→3)-glucan. The presence of both glucans was proved by hydrolyses with specific β-1,3-glucanase and α-amylase. The ability of this spent yeast bioemulsifier to form stable emulsion with vegetable oils suggests potential application as additive in foods and pharmaceuticals.

CHAPTER 5

SURVIVAL OF ENCAPSULATED PROBIOTIC *LACTOBACILLUS PLANTARUM* D6SM3 WITH BIOEMULSIFIER DERIVED FROM SPENT YEAST IN SIMULATED GASTROINTESTINAL CONDITIONS

5.1 Abstract

The effect of microencapsulation with three kinds of emulsifier (tween 80, gum arabic and bioemulsifier extracted from spent yeast) on the survival of *Lactobacillus plantarum* D6SM3 in simulated gastrointestinal tract and on their stability during storage at 4°C and room temperature was investigated. The survival of all encapsulated cell treatments in simulated gastric juice was higher than free cells at both pH 2.5 and 3.0. The survival of free and microencapsulated cell in simulated small intestinal juice after sequential incubation in simulated gastric juice was also studied. They retained their viability with a little reduction (less than 1.0 log cfu.ml^{-1}). The viability of the free and encapsulated cells showed a gradual decline throughout the storage period at 4°C. Their viability decrease was from 10 log cfu·ml^{-1} to about 8 log cfu.ml^{-1}. But the viability rapidly declined at room temperature. Their viability decrease was from 10 log cfu.ml^{-1} to about 4 log.cfu ml^{-1}.The droplet size distribution of microencapsulated cells was compared between those with and without an emulsifier by using the Laser diffraction method. The particle size and polydispersity value of microencapsulated cells were controlled better in emulsion with emulsifier added. The surface of encapsulated cells with emulsifier added exhibited smoother characteristics than those without emulsifier.

5.2 Introduction

Probiotics have been defined as “live microbial feed supplements that have beneficial effects on the host by improving their intestinal microbial balance” (Fuller, 1992). In order to bring about positive health effects, lactic acid bacteria (LAB) have to resist gastric juice and bile salts. However, studies have indicated that the bacteria may not survive in sufficient numbers after the LAB pass through the gastrointestinal tract (Hamilton-Miller, 1999). International standards recommended that probiotics should be at the level of 10^6-10^7 CFU/g in the product at the time of consumption (Ouwehand and Salminen, 1998). However, many products fail to meet these standards when they are consumed (Shah *et al*., 1995).

Attempts have been made to improve the survival of LAB. Technologies that can protect the viability of probiotics during storage and gastrointestinal transit are highly sought after. Microencapsulation in alginate beads is one method of improving their viability by retaining probiotics within a polymer membrane or matrix to reduce cell injury or cell loss. Two widely used methods of encapsulation are the extrusion and emulsion techniques. Emulsion technique have a smaller encapsulation cell, and provides advantages with regard to minimizing adverse impacts on texture and how they feel in the mouth when incorporated into food. The size of the beads is controlled by the speed of agitation (Krasaekoopt *et al*., 2003). In some cases emulsifiers are added to form a better emulsion, because the emulsifier lowers the surface tension and results in smaller spheres. This can also provide more control over the particle size and good polydispersity (Adamson, 1982). The most common emulsifier used is Tween 80 (Sheu *et al.,* 1993). Moreover, the type of emulsifier has been reported to influence the shape of capsules (Krasaekoopt *et al*., 2003).

The yeast strain *Saccharomyces cerevisiae* is normally used for alcohol fermentation. Generally, local Thai producers directly distill palm sugar wine without separate yeast cells to obtain spirit. After distillation a huge amount of waste containing yeast cells is discharged. This causes environmental problems. Bioemulsifier extracted from *S. cerevisiae* has been shown to be an effective emulsifier (Cameron *et al*., 1988). Bioemulsifier with hydrophilic glucose polymers

covalently attached to the protein backbone provides the bioemulsifier with the amphiphilic structure common to surface active agents and effective emulsifier (Cooper and Goldenberg, 1987). An bioemulsifier obtained as a by-product of the wine or brewer's industry, is readily available, has a biodegradable nature, is not toxic and large scale production is possible. It makes it possible to produce value-added by-products (Torabisadeh *et al*., 1996). Since *S. cerevisiae* is edible and is used in the manufacture of food and beverage products, it is assumed that a bioemulsifier would be non-toxic and generally recognized as safe (GRAS) (Cameron *et al*., 1988).

The study reported in this paper evaluated the efficacy of a bioemulsifier extracted from spent yeast obtained from Thai traditional liquor distillation as an emulsifier. It was compared with commercially emulsifier to assess whether it increased the survival rate of encapsulated probiotics LAB when exposed to simulated gastrointestinal conditions. In addition, the stability of the encapsulated cells during storage over 28 days at refrigerated and room temperatures were also evaluated.

5.3 Materials and Methods

5.3.1 Bacterial strain, growth condition and preparation of cell suspensions

Probiotic lactic acid bacterial strain *Lactobacillus plantarum* D6SM3 isolated from a fermented shrimp (Hwanhlem *et al*., 2010) was the best strain that resistant to simulated gastric juice at pH 2.5 and 3.0 also resistant to 3 $mg.ml^{-1}$ of pancreatin and ox bile salts at pH 8.0. This strain was cultured in MRS broth (Labscan Asia Co., Ltd., Thailand) at 37°C. It was checked for purity and maintained in MRS broth supplemented with 25% (v/v) glycerol at -20°C. For routine analysis, the strain was sub-cultured twice in MRS broth for 24 h at 37°C.

5.3.2 Extraction and partial purification of bioemulsifier

Distillate residues were obtained from local distillery in Songkhla Province, Thailand. It was centrifuged (SCR20B, Hitachi, Japan) at 6000×*g* for 10 min at 4°C. The yeast cells were washed twice in normal saline. Twenty percent (w/v) yeast cells were suspended in distilled water containing 0.1 M potassium citrate and 0.02 M potassium metabisulfite. The pH of the suspension was adjusted to 7 with 1M

NaOH. The cell suspension was autoclaved (121°C) for 30 min (Dikit *et al*., 2010). The resulting suspensions were centrifuged at 6,000×*g* for 10 min at 4°C.

The supernatant was retained and mixed with five times the volume of chilled ethanol, and incubated overnight at 4°C for complete precipitation. The suspension was centrifuged at 6,000×*g* for 10 min at 4°C. After centrifugation, the supernatant was discarded and the precipitate was washed twice with cold ethanol. The precipitate was dried by rotary evaporator and freeze dried (Barriga *et al*., 1999). Crude bioemulsifier (10g) from spent yeast was dissolved in 50 ml distilled water and dialysed (8,000 dalton molecular weight cut-off) against distilled water overnight. Finally, the partial purified bioemulsifier was lyophilized.

5.3.3 Cell preparation for microencapsulation

The 8% starter culture of *L. plantarum* D6SM3 were inoculated in 50 ml MRS broth and incubated at 37˚C for 24 h to obtain a cell density of about 10^{10} cfu.ml^{-1}. Harvesting of the cells was done by centrifugation at 6,500×*g* for 20 min at 4°C. Cell pellets were washed twice with sterile normal saline. Washed cells were then suspended in two mixtures of 1 ml of sterile normal saline, one without and one with 0.2% emulsifier (bioemulsifier, tween 80 and gum arabic) and stored at 4°C until used.

5.3.4 Microencapsulation and enumeration of microencapsulated LAB

L. plantarum D6SM3 was encapsulated in sodium alginate mixture by using a modified method of Sheu *et al.* (1993). Washed cells were prepared for encapsulation by the emulsion technique. A washed cell suspension was added to 20 ml of 2% sodium alginate and the mixture was then emulsified into 20 ml of palm oil (Morakot, Morakot industry Co. Ltd., Thailand). The emulsion was produced through vigorous stirring for 5 min until it was creamy. A solution of 0.1 M calcium chloride (100 ml) was then added quickly along the side of the beaker and vigorously continuous stirring again for 5 min. The mixture was allowed to stand for the sodium alginate beads to separate and settle at the bottom of the calcium chloride layer for 30 min. The oil layer was then removed and the beads were collected by centrifugation at 6,500×*g* for 20 min at 4°C. The beads were then rinsed by using sterile filter paper

and stored in peptone saline (1 $g.l^{-1}$ peptone, 8.5 $g.l^{-1}$ sodium chloride) pending further analysis.

The microencapsulated LAB was enumerated as described by Kailasapathy, (2006) and Annan *et al.* (2007). The encapsulated bacteria in the microcapsules were released by using 1.0 g of a filtered microcapsule and were re-suspended in 9.0 ml of PBS buffer (pH 7.6) in a sterile plastic bag. It was homogenized for 10 min to allow complete release of the bacteria from alginate capsules. The homogenized samples were diluted to appropriate concentrations and plated on MRS agar (Labscan Asia Co., Ltd., Thailand). The plates were incubated for 24 h at 37°C and the encapsulated bacteria were enumerated as $cfu.ml^{-1}$.

5.3.5 Survival assay of free and microencapsulated *L. plantarum* D6SM3 in simulated gastric and intestinal juices

Simulated gastric juice was prepared by dissolving pepsin (Fluka, USA) in phosphate buffer to a final concentration of 3 $mg.ml^{-1}$ and the pH adjusted to 2.5 and 3.0 with 6M HCl. The mixture was sterized by membrane filtrate through a membrane (0.45 µm, Sartorius, Germany) (Maragkoudakis *et al*., 2006).

Simulated intestinal juice was prepared based on the method of Krasaekoopt *et al*. (2004). Pancreatin (Sigma, Germany) and ox bile salts (Difco, USA) were suspended in phosphate buffer to final concentrations of 3 $mg.ml^{-1}$and 3 $mg.ml^{-1}$, respectively. The mixture was adjusted to a pH of 8.0 with 6 M NaOH and then sterile filtered.

Washed cell suspensions of *L. plantarum* D6SM3 (1.0 ml) or 1 g of microencapsulated cells were added to either 9 ml of tempered (37°C) simulated gastric juice or simulated intestinal juice. There were incubated at 37°C for 4 h (Maragkoudakis *et al*., 2006).

The survivals of the free cells and encapsulated *L. plantarum* D6SM3 before and after exposure to simulated gastric juice and simulated small intestinal juice was determined by plating in MRS agar. The plates were incubated at 37°C for 24 h (modified from Madureira *et al*., 2005).

5.3.6 Survival assay of free and microencapsulated *L. plantarum* D6SM3 after sequential incubation in simulated gastric juice and in simulated small intestinal juice

One gram of the encapsulated *L. plantarum* D6SM3 and 1 ml of non-encapsulated probiotic sample were incubated in 9 ml of simulated gastric juice (3 $mg.ml^{-1}$ pepsin, pH 2.5) at 37°C for 4 h. Microencapsulated beads and non-encapsulated probiotic samples in simulated gastric juice were then centrifuged at 6,500×*g* at 4°C for 20 min and washed with 0.85% sodium chloride. The free cells and capsules obtained were re-suspended in 9 ml of simulated small intestinal juice (3 $mg.ml^{-1}$ pancreatin, 3 $mg.ml^{-1}$ ox bile salts, pH 8.0) at 37°C for 4 h. The survival of the free cells and encapsulated probiotics before and after exposure to small intestinal juice for 4 h was determined by plating in MRS agar. Plating was incubated at 37°C for 24 h (modified from Madureira *et al*., 2005).

5.3.7 Viability of free and microencapsulated *L. plantarum* D6SM3 during storage

The 1 ml of free and 1 g of microencapsulated *L. plantarum* D6SM3 in 9 ml of peptone saline were stored at room temperature and 4°C. They were assessed periodially for 0, 1, 2, 3 and 4 weeks to determine viability during the storage period. The enumeration of the viable probiotic bacteria in each experiment was performed as previously described.

5.3.8 Physical examination of microcapsules

5.3.8.1 Size analysis

The droplet size distributions (DSD) of the microcapsules was determined by using a laser diffraction method (Mastersizer, 2000) as described by Hayati *et al*. (2007). Distilled water was used as the dispersant for the determination of the size distribution. The software used a reflective index of dispersant RI 1.33 (water) to calculate the Dispersion Index (Span) by span = d(90)-d(10)/d(50). The d(10), d(50) and d(90) values are size values corresponding to the cumulative distribution at 10%, 50% and 90%, respectively. Thus, the d(10) represents a size value below which 10% of the cumulative distribution is present. Microcapsules were

introduced into the sample presentation unit until the concentration reached the optimum one, as indicated by the instrument.

5.3.8.2 Morphology

The morphology of the external structure of the encapsulated cell was observed by using the scanning electron microscope (SEM, JSM-5800LV, JEOL, Tokyo, Japan). This was analyzed at 10 kV acceleration voltage.

5.4 Results and Discussion

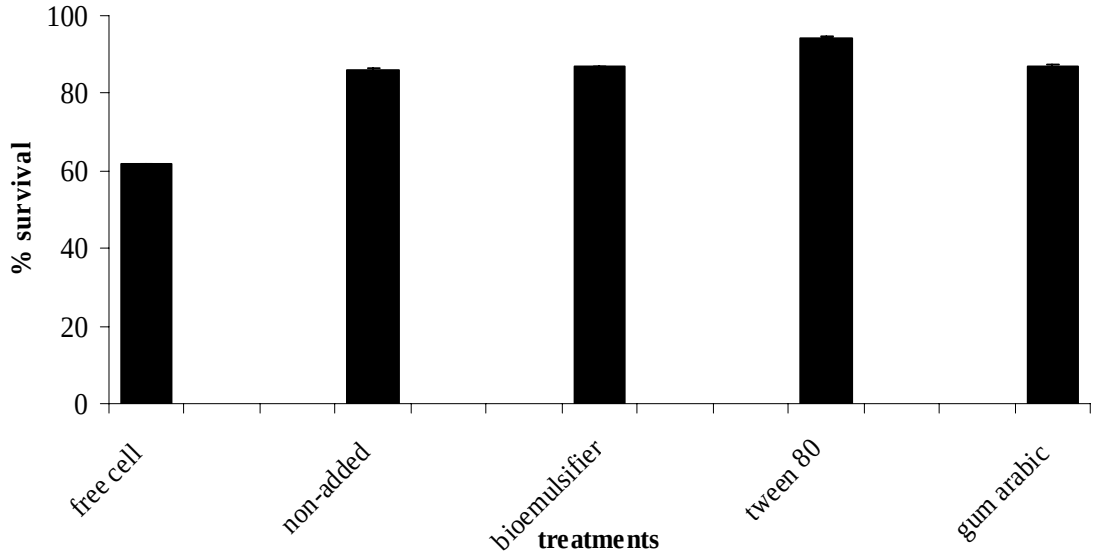
5.4.1 Survival of free and microencapsulated *L. plantarum* D6SM3 in simulated gastric juice

The survival of the free and microencapsulated probiotic LAB in simulated gastric juice for 4 h, reflecting the time spent by food in the stomach, is shown in Figure 28. Food remained in the stomach for 2 to 4 h, while liquids empty from the stomach in only about 20 min (Annan *et al*., 2007). The pH of the simulated gastric juice affected the survival of both free cells and the encapsulated probiotic LAB. The microencapsulated probiotic LAB survived well in simulated gastric conditions compared to the free probiotic bacteria cells. As a result, a densely cross-linked gel structure was formed. The alginate mixture stays structurally stable in low acid environments. However as the pH is falls below the pKa values of mannuronic (3.6) and guluronic acid (3.7) the alginate is converted to alginic acid with the release of calcium ions and the formation of a more dense gel (Doumeche *et al*., 2004).

This suggested that better protection of bacterial cells was achieved with the microencapsulated bacterial cells than with the free cells. The microencapsulation technique protected the cells from the effect of both the pH and pepsin enzyme in simulated gastric juice. The survival of probiotic LAB in all microencapsulated treatments (without emulsifier and with added tween 80, bioemulsifier and gum arabic) was higher than in the free cells at the tested pH (Fig. 28). The survival of probiotic LAB in simulated gastric juice at pH 3.0 was higher than survival at pH 2.5. There was about a 4-log decreased in viable cell of free cells in pH 3.0 and no survival was observed when the pH of simulated gastric juice decreased to 2.5.

In addition, it was found that among the three types of emulsifier, tween 80 exhibited higher survival than other types of emulsifier at pH 3.0. At pH 2.5, adding emulsifier resulted in remarkably higher survival than did the encapsulated cells without emulsifier. It also showed that bioemulsifier and tween 80 were better than gum arabic at pH 2.5 as in Figure 28b. Bioemulsifier gave a similar result with tween 80 because bioemulsifier not only act as an emulsifier in the system but also may form extramembrane with alginate. Both properties of bioemulsifier could enhance stability of the bead in acid condition like tween 80. Despite the suitability of alginate as the encapsulation matrix material, gel encapsulation in alginate has some limitation. A cross-linked alginate matrix system at very low pH is reported to undergo a reduction in alginate molecular weight causing a faster degradation and release of active ingradients (Krasaekoopt *et al*., 2006). Therefore special treatment, such as coating the bead with extramembrane, could apply in order to improve the properties of encapsulated beads. Coated beads not only prevent cell release but also increase mechanical and chemical stability (Krasaekoopt *et al*., 2003). This result suggested that the microencapsulation technique could protect *L. plantarum* D6SM3 in the gastro environment. This result was in agreement with previous studies that showed that microencapsulation provided better protection of cells in an acid environment (Jacobsen *et al*., 1999; Dunne and Mahony, 2001; Godersky *et al*., 2003; Krasaekoopt *et al*., 2003; Lee *et al*., 2004; Musikasang *et al*., 2009) As a result, alginate encapsulation may provided significant protection of cells from the acidic condition of simulated gastric juice, as alginate gel are stable in the low pH solution.

(a)



(b)

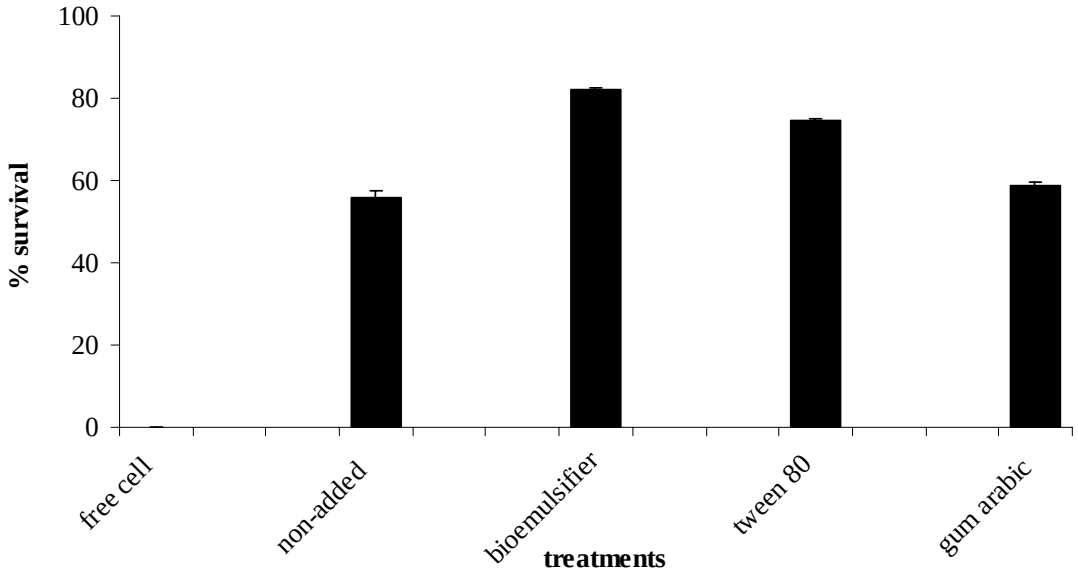


Figure 28. Survival of free and encapsulated probiotic *Lactobacillus plantarum* D6SM3 after exposure to simulated gastric juice at (a) pH 3.0 and (b) pH 2.5 for 4 h. Bars represent the standard deviation from triplicate.

5.4.2 Survival of free and microencapsulated *L. plantarum* D6SM3 in simulated intestinal juice

Bile salt tolerance is considered to be one of the essential properties for LAB to survive in the small intestine. Survival of free cells and encapsulated probiotic LAB after exposured to 0.3% bile salt for 4 h, reflecting the time of food spent in the small intestine, is shown in Figure 29. It was found that the survival of non-encapsulated and encapsulated probiotic LAB did not show a significant difference ($p>0.05$). It was noted that the survival of the non-encapsulated and encapsulated *L. plantarum* D6SM3 was reduced in some extent. They were resistant to pancreatin and oxgall bile salts. Even after 4 h of exposure to the simulated intestinal juice at pH 8.0 they retained their viability with a little reduction (less than 1.0 log cfu.ml^{-1}). Bile salts did not affect *L. plantarum* D6SM3 since this strain was screened previously for probiotic properties, such as acid and bile salt tolerance (Hwanhlem *et al*., 2010). Except using gum arabic as emulsifier the viability reduced about 2 log cfu.ml^{-1} due to the chemical properties of gum arabic. The quality of gum arabic available to act as an emulsifier becomes limiting. Gum arabic also composed of a complex mixture of the calcium, magnesium and potassium salts of arabic acid that effect unstable of the bead (Kim *et al*., 1996). Because calcium alginate are chemically unstable when chelators such as phosphate and citrate and non-gelling cations such as sodium or magnesium ions are present (Lee *et al.*, 2004). The structure of the bead is weakened that cause rapidly release of the cell.

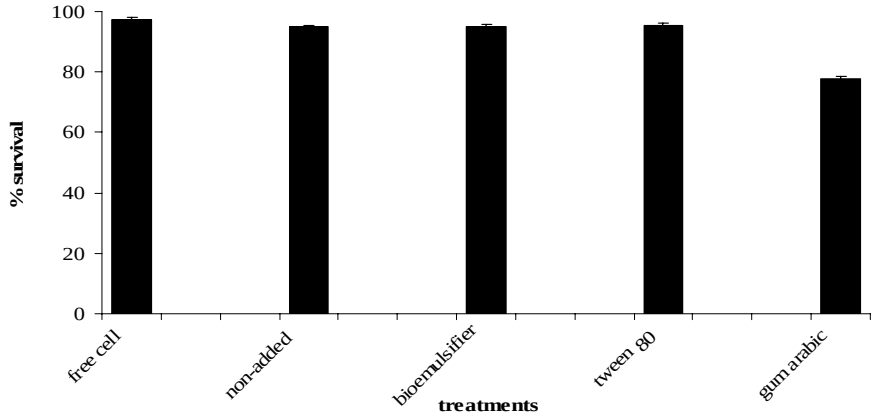
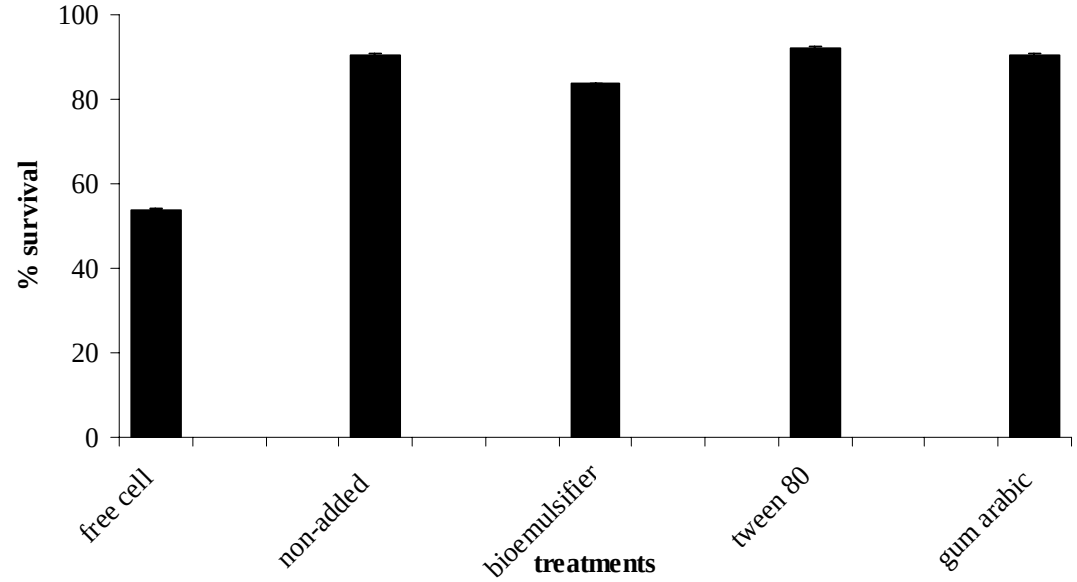


Figure 29. Survival of free and encapsulated *Lactobacillus plantarum* D6SM3 after exposure to simulated intestinal juice at pH 8.0 for 4 h. Bars represent the standard deviation from triplicate.

5.4.3 Survival assay of free and microencapsulated *L. plantarum* D6SM3 after sequential incubation in simulated gastric juice and in simulated small intestinal juice

Several articles were studied on acid and bile tolerance. An important difference in this present study is that bacterial cells were passed through acid and bile salt consecutively. This imitated the real situation in the gastrointestinal condition. The survival of free cells and encapsulated *L. plantarum* D6SM3 in simulated intestinal juice after being incubated with simulated gastric juice is shown in Figure 30. The same results are shown in either an acid or bile salt condition. The survival of probiotic LAB in simulated gastric condition at pH 3.0 was higher than survival at pH 2.5. There was about a 4-log decreased in the viable cells with free cells at pH 3.0, and no survival was observed when pH decreased to 2.5. However, encapsulated cell using tween 80 as emulsifier showed the highest survival due to it had higher stability of the bead. A number of factors would likely affect the ability of the material to function as emulsifier, i.e., emulsifier concentration, proportion of dispersed and dispersion phases, processing conditions and properties of the material to be encapsulated. These factors control their ability to interact via hydrogen bonding, van der waals forces, dipole and electrostatic interactions, hydrophobic association and formation of covalent disulfide bonds are believed to effect their emulsification properties (Kim *et al*., 1996). Tween 80 is the most common emulsifier used may showed higher stable after sequential incubation in simulated gastric juice and in simulated small intestinal juice. The microencapsulation technique protected the cells from harsh conditions, that is an acidic condition. Sequential incubation affected the survival of free cells in both conditions and even microencapsulated cells in exposed to simulated gastric juice at pH 2.5 (Fig. 30). We can improve the stability of the bead using bioemulsifier as an emulsifier by optimizing the conditions that used in the cell microencapsulation process, such as proportion of dispersed and dispersion phases, co-encapsulated with other material that could more stabilized the bead. These may sufficient to produce stable bioemulsifier emulsions.

(a)



(b)

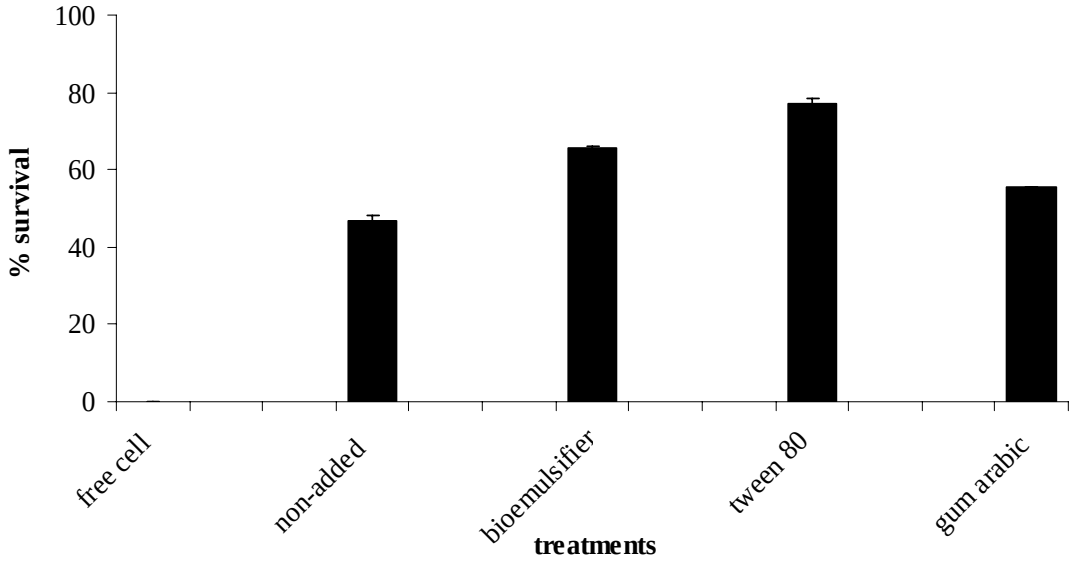


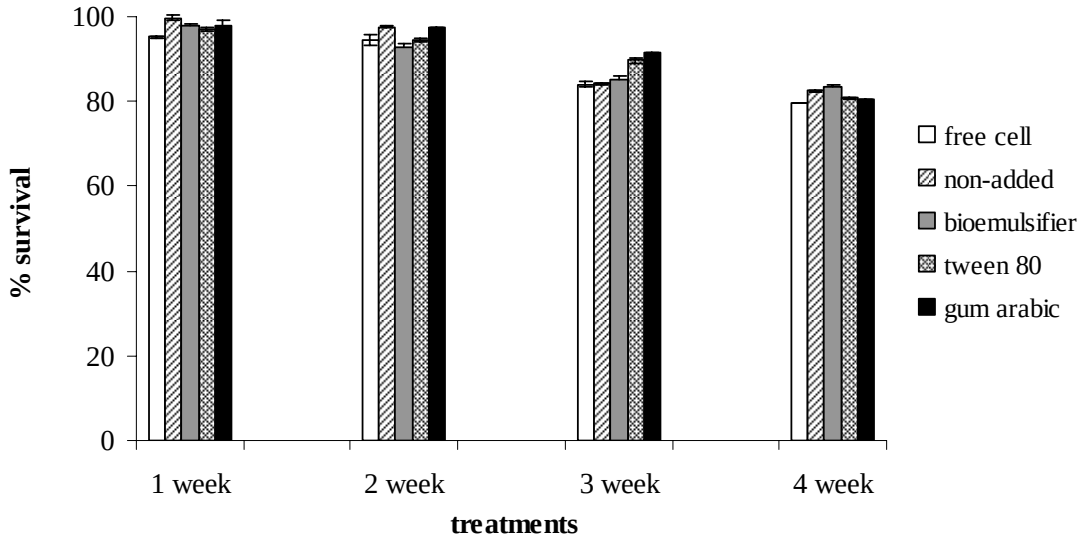
Figure 30. Survival of free and encapsulated *Lactobacillus plantarum* D6SM3 after exposure to simulated intestinal juice after sequentially incubated with simulated gastric juice (a) pH 3.0 (b) pH 2.5 for 8 h. Bars represent the standard deviation from triplicate.

5.4.4 Viability of free and microencapsulated *L. plantarum* D6SM3 during storage

Microencapsulation of probiotic bacteria has been a common practice for expanding their shelf life (Krasaekoopt *et al*., 2003). This study evaluated the stability of *L. plantarum* D6SM3 both free and encapsulated in sodium alginate during 4 weeks of storage in refrigerated and room temperature. The number of viable cells of free and encapsulated cell declined gradually throughout the storage period at 4°C. The decrease was from 10 log cfu.ml^{-1} to about 8 log cfu.ml^{-1} after 4 weeks of storage (Fig. 31a). When free cells and four kinds of emulsifier were added to encapsulated cell were compared, it was found that the survival rate of the encapsulated cells was higher than the free cells. However, at room temperature the number of viable free and encapsulated cells declined more rapidly than at 4°C throughout the storage period. The decrease was from 10 log cfu.ml^{-1} to about 4 log cfu.ml^{-1} after 4 weeks of storage (Fig. 31b).

In addition, the survival of the encapsulated cells was much higher than that of free cell except when gum arabic was added as an emulsifier. The survival rate of the encapsulated cells with gum arabic after 2 weeks was not different to that of free cells. The survival rate was rapidly reduced by adding bioemulsifier as an emulsifier after storage for 4 weeks. It was found that the survival rate of encapsulated cells with both gum arabic and bioemulsifier, was lower than others after 4 weeks of storage. This was because emulsifier used have different properties. Gum arabic and bioemulsifer are oil-in-water emulsion whereas tween 80 is an water-in-oil emulsion. Proportion of oil using in the process effect the quality of gum arabic and bioemulsifier available to provide the structural of the encapsulated bead to function as an emulsifier. The content of the oil may not suitable for oil-in-water emulsion of gum arabic and bioemulsifier. Accordingly, it could be effect to the stability of the emulsion after 4 weeks of adding gum arabic and bioemulsifier as emulsifier. However, several studies have shown that the survival of microencapsulated bacteria was improved in alginate microparticles compared with non-encapsulated bacteria during the storage period (Sultana *et al*., 2000; Hansen *et al*., 2002; Lee *et al*., 2004). Morever, several environmental factors, i.e., temperature, pH, ionic composition and ionic strength could affect the ability of the material to function as an emulsifier.

(a)



(b)

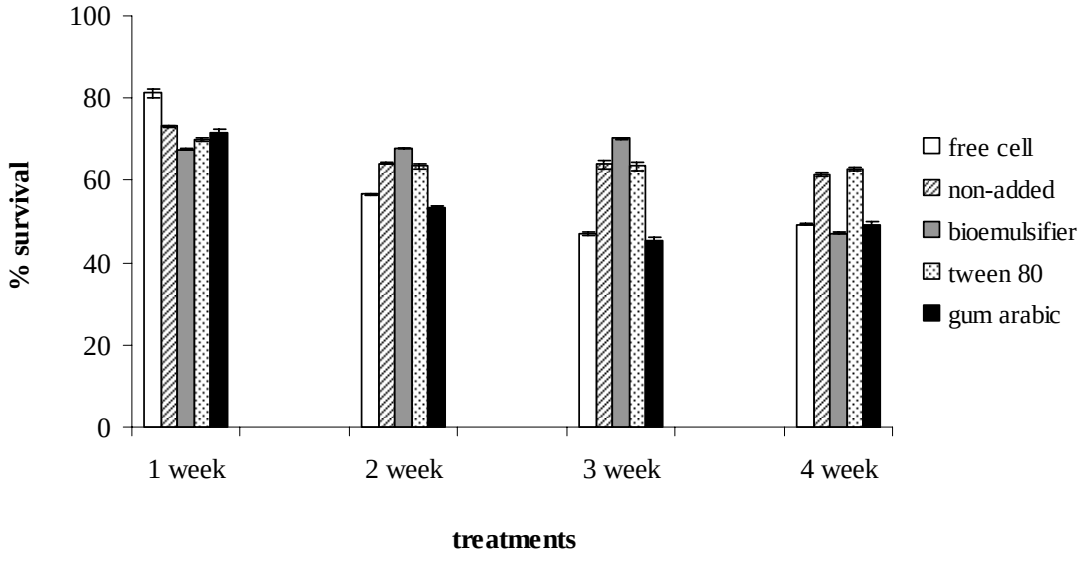


Figure 31. Temperature storage for survival rate of encapsulated *Lactobacillus plantarum* D6SM3 before and after exposure to simulated gastric juice (a) at 4°C (b) at room temperature. Bars represent the standard deviation from triplicate.

5.4.5 Physical examination of microencapsulated LAB

The droplet mean diameters of d(10), d(50), d(90) and the span is presented in Table 21. The droplet size of microencapsulated beads with and without emulsifier (bioemulsifier, tween 80 and gum arabic) were compared. There were 3 indications of the distribution. The d(10) indicates that there are about 10% of smaller droplets (µm) in the distribution. The d(50) shows that there are half of all droplets (µm) in the distribution. The d(90) shows that there are about 90% of smaller droplets (µm) in the distribution. In addition, the span indicates the width of the distribution regardless of the median size (Palazolo *et al*., 2004). The particle size where the cumulative distribution is 50% is known as the median droplet diameter ($d_{v,0.5}$). The beads without emulsifier added had a $d_{v,0.5}$ with 50% of the particles under 237.65 µm. This must be compared with those tween80, gum arabic and bioemulsifier added as emulsifier as these showed sizes of 358.59 µm, 338.91 µm and 241.72 µm respectively (Fig. 32). This showed that the smallest particle size was obtained from the beads without emulsifier. However, the span was highest that showed the highest polydispersity of the bead size.

The size of the beads formed by this technique was in the same range as that of Krasaekoopt *et al*. (2003) which were 25 µm to 2 mm. However, the size of the beads using the emulsion method depended on the speed of agitation and the type of emulsifier used. The smallest bead size was obtained when the mixture without emulsifier was used. Better emulsion resulted when emulsifier was used since the emulsifiers lower the surface tension which results in smaller spheres.

Table 21. Droplet mean diameters and dispersity Index (Span) of microencapsulation beads.

Samples	d(10)* µm	d(50)* µm	d(90)* µm	Span
Non-added emulsifier	57.857	237.652	524.801	1.965
Added tween80	100.747	358.589	698.191	1.666
Added gum arabic	81.017	338.914	699.533	1.825
Added bioemulsifier	39.090	241.715	501.783	1.914

* The experiments were done in triplicate and results were reported as the average from triplicate determinations.

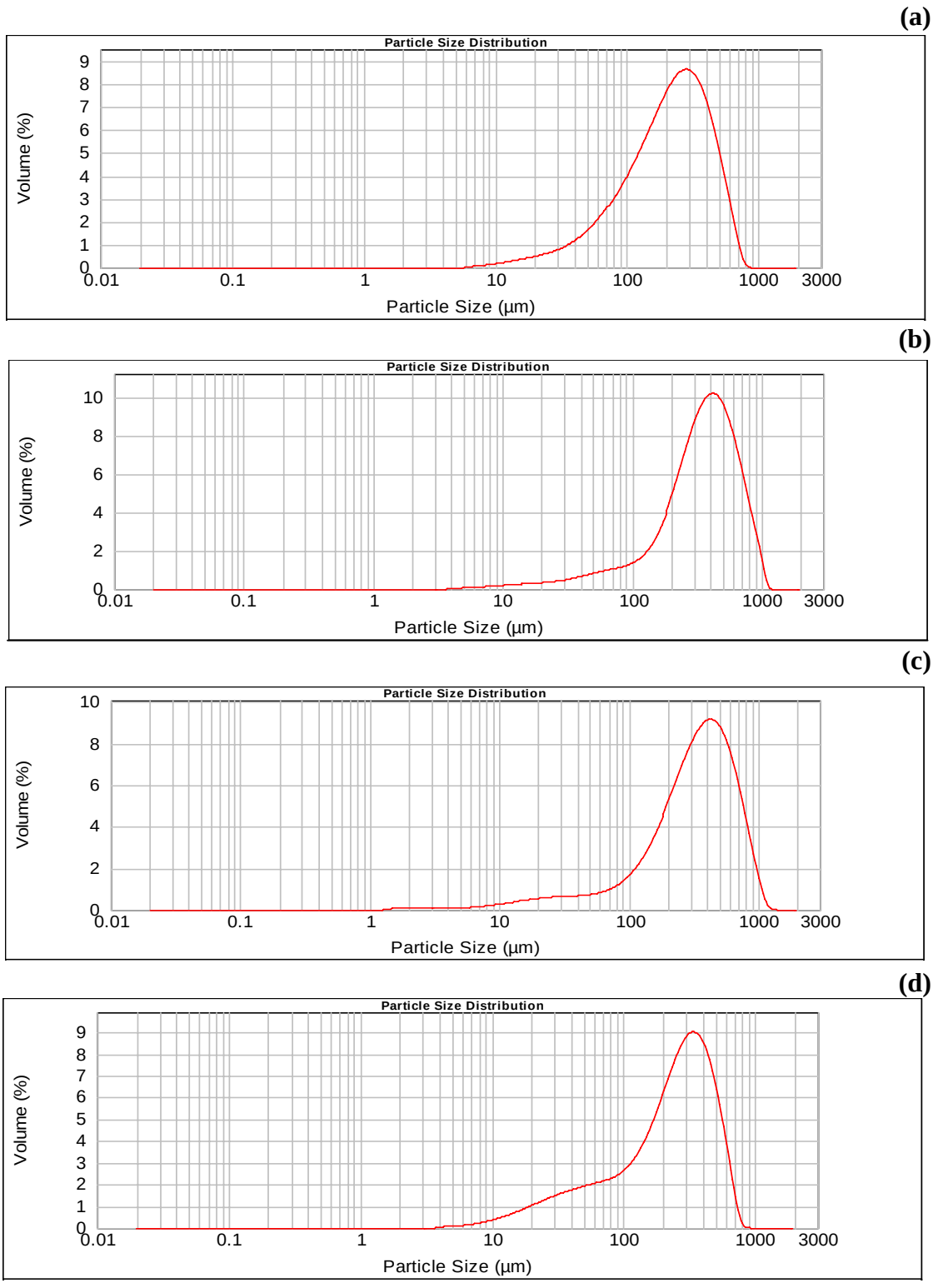
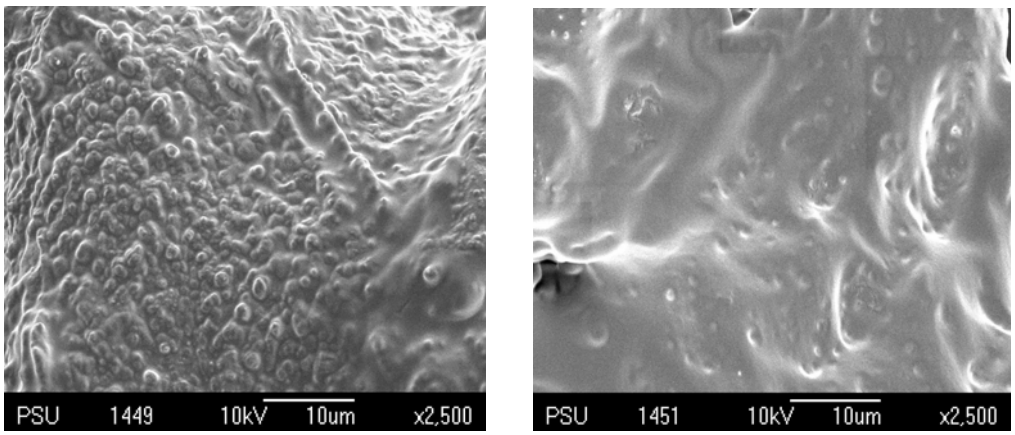


Figure 32. Sized distribution of encapsulated cell without emulsifier (a), with tween 80 (b), with gum arabic (c), and with the spent yeast bioemulsifier (d) as an emulsifier.

A morphological analysis of the freeze-dried microencapsulated LAB was undertaken by scanning electron micrographs. This showed different appearance of the one without emulsifier compared with the three with different emulsifiers. It revealed that the surface of those with emulsifiers added (bioemulsifier, gum arabic and tween 80) had microcapsules that were smoother than the one without emulsifier (Fig. 33). The one without emulsifier showed microcapsules with an irregular surface. It showed that emulsifiers could emulsify the oil droplets better than if emulsifier was not added. Consequently, better emulsion capsules formed when emulsifier was used since the emulsifiers lower the surface tension.

However, the texture of freeze-dried microcapsules was changed. Kwok *et al.* (1991) reported that the wrinkled surface was formed due to the loss of water content during the freeze-dried process. Moreoever, observing the microcapsules by scanning electron micrographs indicated that the droplets were much more flocculated than with microcapsules without emulsifiers. Scanning microcapsules with electron micrographs showed that the lactic acid bacteria remained trapped within the alginate material (data not shown). This was because the microencapsulated ones were washed before analysis. Lactic acid bacteria cells were not visible on the surface of the capsules. However, compared with those without emulsifier, adding emulsifier not only effect the form of microcapsules, it also increased the survival of *L. plantarum* D6SM3. From the results, bioemulsifier from spent yeast is the alternative bioemulsifier for using in the food industry.

(a) (b)



(c) (d)

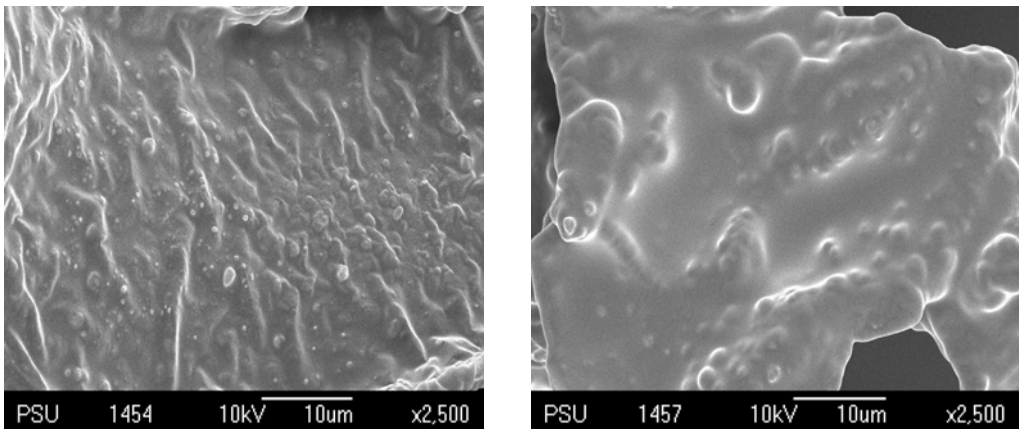


Figure 33. (SEM) image of non-added emulsifier (a), added tween 80 (b), added gum arabic (c), and added the spent yeast bioemulsifier (d).

5.5 Conclusion

Microencapsulation through emulsion techniques is an effective way to increase the survival of probiotics bacteria as they pass through the gastrointestinal conditions of the human stomach. Bioemulsifier from spent yeast could be used as an emulsifier in the microencapsulation process. Adding emulsifiers when using microencapsulation techniques can improve the survival of probiotic bacteria.

CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

1. The spent yeast produced as a waste from the local liquor distillation could provide a source of raw material for the mass production of bioemulsifier. It can be extracted by a low-cost method, using water-soluble substrates (citrate buffer). As a result, this extraction procedure is simple and suitable for large-scale production and utilizing the abundant waste from distillery. The emulsion activity of the bioemulsifier was similar to those of commercial emulsifiers (lecithin and gum arabic). Palm oil-in-water emulsions were stabilized over a broad range of conditions, from pH 3-12, with up to 3% (w/v) NaCl and up to 0.1% (w/v) $CaCl_2$ and $MgCl_2$.

2. *Saccharomyces cerevisiae* KA01 was isolated from sugar palm wine obtained from a local brewer in Songkhla Province could also provide a source of raw material for the mass production of bioemulsifier. These findings reveal that the products obtained could be very useful in situations where extreme conditions of temperature, salinity and pH 5-8 are present. The capacity to form emulsions with vegetable oils suggests potential applications as an emulsifying agent in the food industry such as in salad dressing.

3. In contrast to previous published bioemulsifier from yeast cell wall which is mannoprotein, in this study, the purified bioemulsifier from spent yeast obtained from Thai traditional liquor distillation was found as β-glucan-protein complex with average molecular weight (M_W) approximately 1.93×10^5 dalton. This bioemulsifier consisted of approximately 4% proteins and 96% carbohydrates. Glucose was a major monosaccharide with a small proportion of mannose. The FT-IR and NMR spectra revealed its configuration and confirmed the presence of protein and glucan. Finally, the structure presented as β-(1,3)-linked glucose was proved by hydrolysis with a specific β-1,3-glucanase. The ability of this bioemulsifier to form stable emulsions with vegetable oils suggests potential applications as food additives and pharmaceuticals.

4. Bioemulsifier from spent yeast could be applied to used as an emulsifier in the microencapsulation process by comparing with commercial emulsifier, tween 80 and gum arabic. Microencapsulation through using emulsion techniques is an effective way to increase the survival of probiotics bacteria as they pass through the gastrointestinal conditions of the human stomach. Adding emulsifiers when using microencapsulation techniques can improve the survival of probiotic bacteria.

6.2 Future works

1. Oligosaccharide products after hydrolyzed with β-1,3-glucanase should be further investigated by using specific HPLC column for studying branching of this structure.

2. 2-D NMR analysis of purified bioemulsifier should be studied.

3. The application of bioemulsifier in other fields such as antimicrobial and antitumor activities should be studied.

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APPENDIX

APPENDIX A

Preparing culture mediam

1. Yeast malt extract broth (YM medium)

Composition per liter:

Glucose	10	g
Bactopeptone	5	g
Yeast extract	3	g
Malt extract	3	g

Preparation of medium:

Add components to distilled water and bring volume to 1 l. Mix thoroughly and distribute to tube or flask, sterile for 15 min at 121°C.

2. Acetate agar

Composition per liter:

Potassium acetate	9.8	g
D-glucose	1.0	g
NaCl	1.2	g
$MgSO_4 \cdot 7H_2O$	0.7	g
Yeast extract	2.5	g
Agar	20	g
Distilled water	1000	ml

Preparatiom of medium:

Add components to distilled water and bring volume to 1 l. Gently heat and bring to boil for melting the agar, sterile for 15 min at 121°C. Mix thoroughly and pour into plate.

3. MRS (de Man Rogosa and Sharp)

Composition per liter:

Dextrose	20	g
Bacteriological peptone	10	g
Beef extract	8	g
Sodium acetate	5	g
Yeast extract	4	g
Dipotassium phosphate	2	g
Tween 80	1	g
Ammonium citrate	2	g
Magnesium sulfate	0.2	g
Manganese sulfate	0.05	g

Preparatiom of medium:

Add 52 g of medium to distilled water and bring volume to 1 l. Mix thoroughly and distribute to tube or flask, sterile for 15 min at 121°C.

APPENDIX B

Preparing chemical analytical

1. Citrate buffer

potassium citrate	0.1	M
potassium metabisulfite	0.02	M
pH	7.0	

2. 0.05 M potassium phosphate buffer

$K_2HPO_4 \cdot 3H_2O$	0.05	M
KH_2PO_4	0.05	M
NaCl	10	g
pH	7.2	

APPENDIX C

1. Protein determination by Bradford's method (Bradford, 1976)

Chemical reagent:

Coomassie brilliant G-250	100 mg
95% ethanol	100 ml
85% phosphoric acid	100 ml
glycerol	100 ml

Bradford's reagent preparation:

1. Dissolved 100 mg of coomassie brilliant blue G-250 in 100 ml of ethanol.
2. Added 100 ml of 85% phosphoric acid and mixed well.
3. Adjusted to 600 ml by distilled water and then mix with 100 ml glycerol.
4. Adjusted to final volume of 1 liter by distilled water and undissolve substrant was filtered out.
5. The Bradford solution was kept overnight at 4°C before use.

Protein determination procedure:

1. Mixed 100 µl of sample with 5 ml of Bradford's reagent.
2. This solution was allowed to stand for 5 min
3. The absorbance was measured at 595 nm.
4. A standard curve was prepared using serum albumin at concentration of 100-1000 µg/ml.

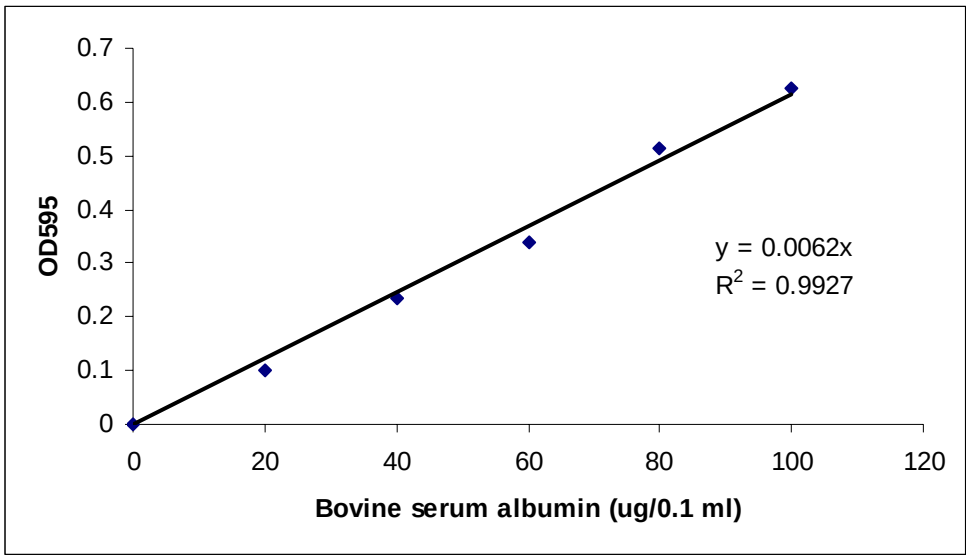


Figure 34. Standard curve of bovine serum albumin.

2. Total sugar determination by Phenol-sulfuric method (Dubois *et al.*, 1956)

Chemical reagent:

5% phenol

concentrated sulfuric acid

Total sugar determination procedure:

1. Mixed 0.1 ml of sample with 0.1 ml of 5% phenol.
2. Then, added 1 ml of concentrated sulfuric into the mixture.
3. This solution was allowed to stand at room temperature for 10 min, immediately vortex-mixed.
4. Leaved stand at room temperature for 30 min.
5. Then, read absorbance at 420 nm. An orange color with intensity proportional concentration will begin to develop immediately.
6. Total sugar of the sample was calculated from standard curve.

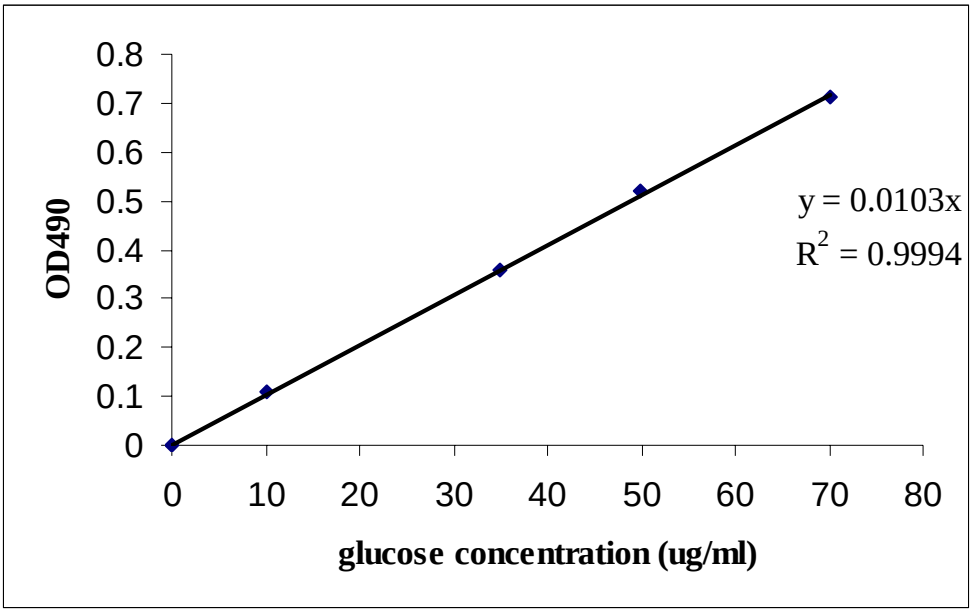


Figure 35. Standard curve of glucose by phenol-sulfuric method.

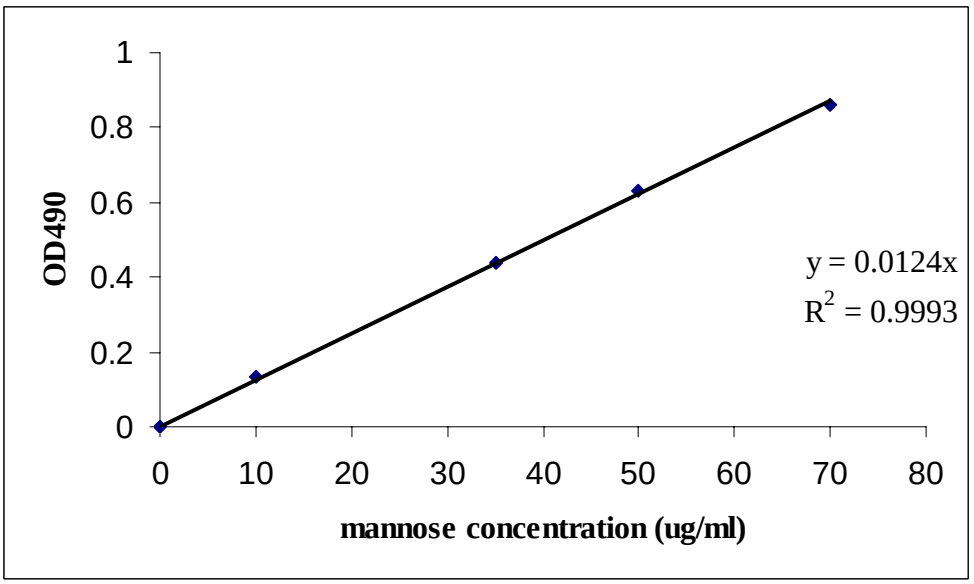


Figure 36. Standard curve of mannose by phenol-sulfuric method.

3. Reducing sugar determination by DNS method (Miller, 1959)

Chemical reagent:

Dinitrosalicylic acid	10.0	g
Na_2SO_3	0.5	g
Na-K tartrate	182.0	g
NaOH	10.0	g
Phenol	2.0	g
Distilled water	1.0	l

DNS solution preparation:

1. Dissolved NaOH in 700 ml of distilled water.
2. Added Na-K tartrate, stirred until well dissolve.
3. Added DNS and stirred continuously.
4. After all DNS well dissolved, added Na_2SO_3 and phenol, respectively.
5. Adjusted to final volume of 1 liler with volumetric flask.
6. Kept DNS solution in brown glass bottle.

Reducing sugar determination procedure:

1. Mixed 0.2 ml of sample with 0.6 ml of DNS solution and boiled for 10 min.
2. Cooled down the sample by immersed the sample tube into cold water immediately, added 1.2 ml of water, mixed well, and measured A_{550}
3. Converted A_{550} to glucose concentration with standard curve.

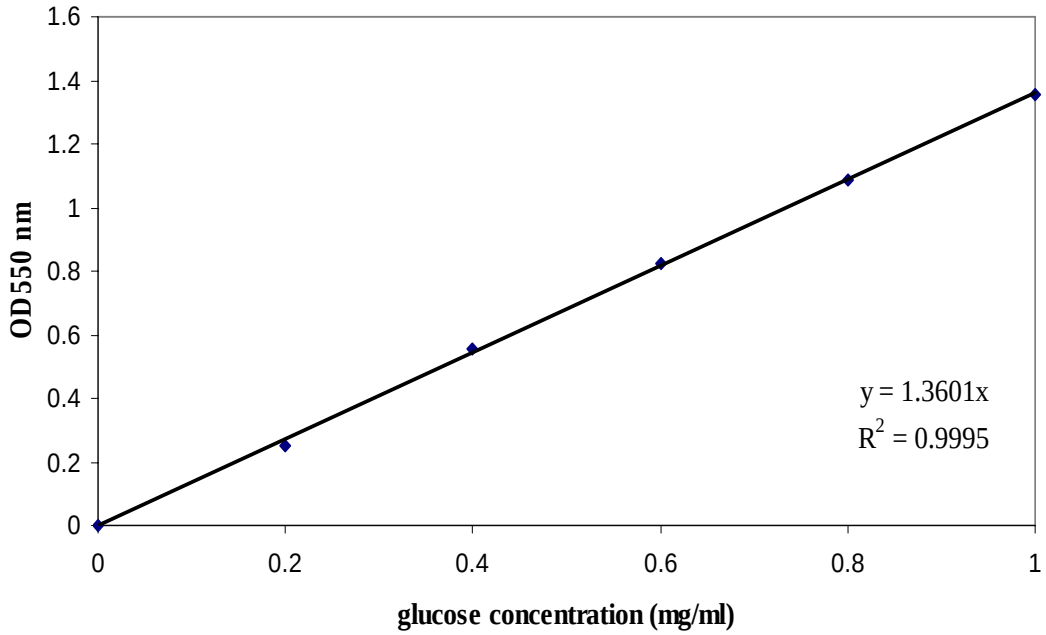


Figure 37. Standard curve of glucose by DNS method.

4. The GPC calibration curve of pullulan standards

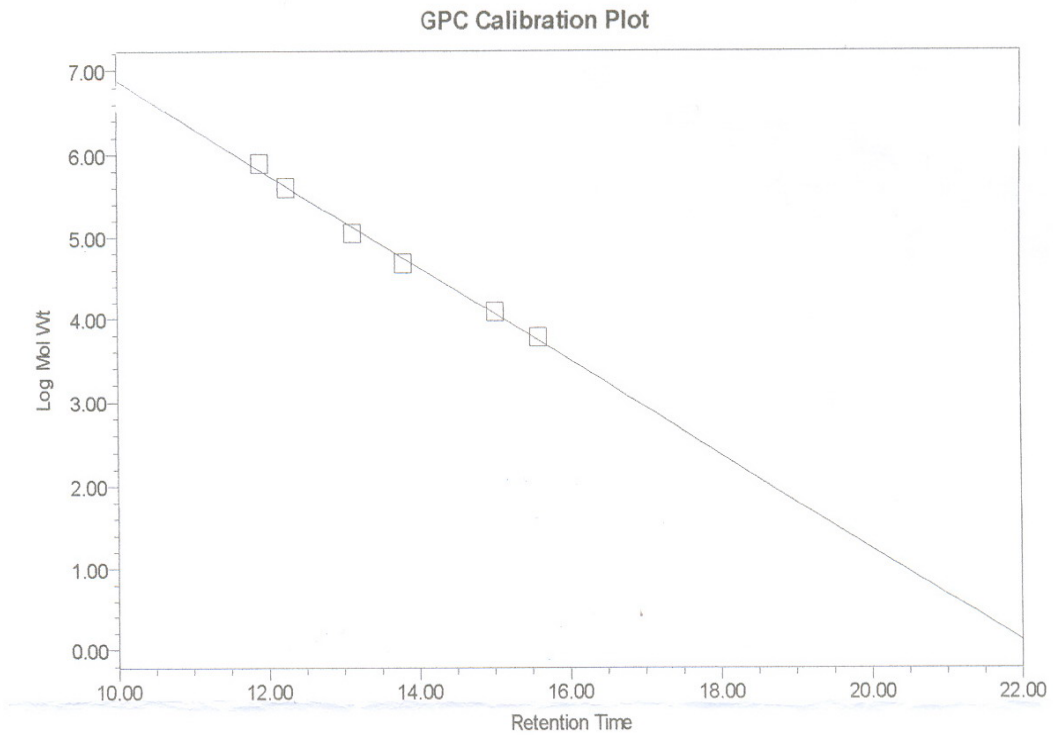


Figure 38. The GPC calibration curve of pullulan standards in the range of 5,900 - 788,000 dalton.

	Mol Wt (Daltons)	Retention Time (min)	Log Mol Wt
1	788000	11.903	5.896526
2	404000	12.251	5.606381
3	112000	13.124	5.049218
4	48000	13.797	4.681241
5	12200	15.018	4.086360
6	5900	15.579	3.770852

5. The HPLC chromatogram of monosaccharide standards

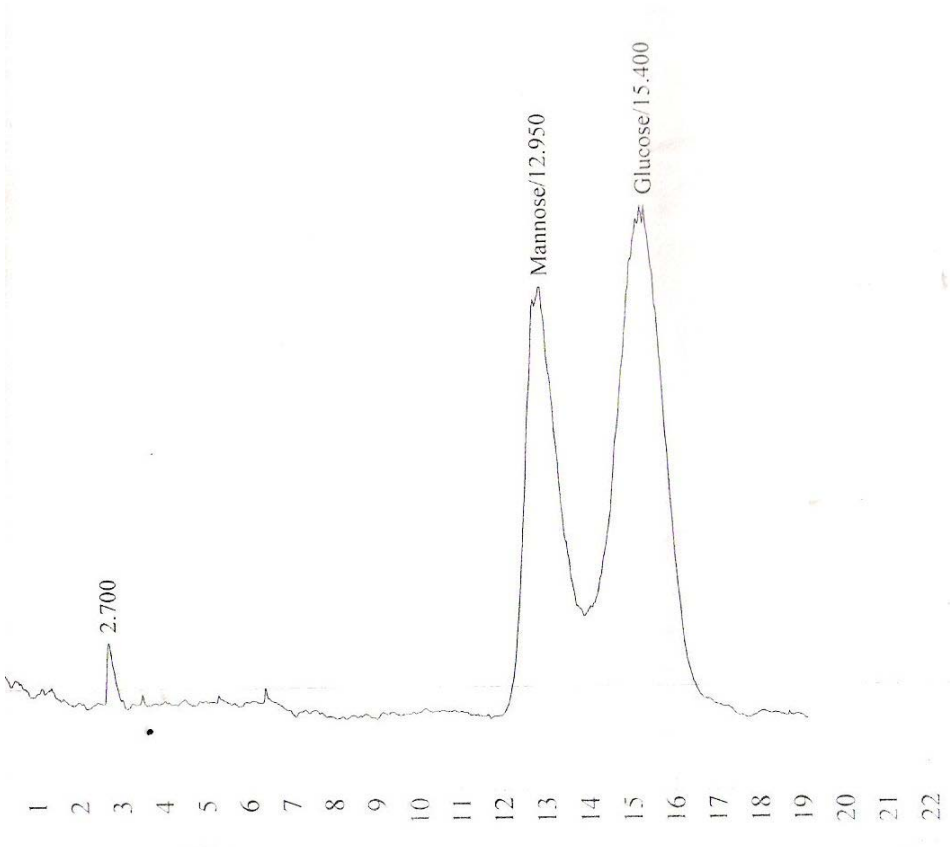


Figure 39. The HPLC chromatogram of monosaccharide standard.

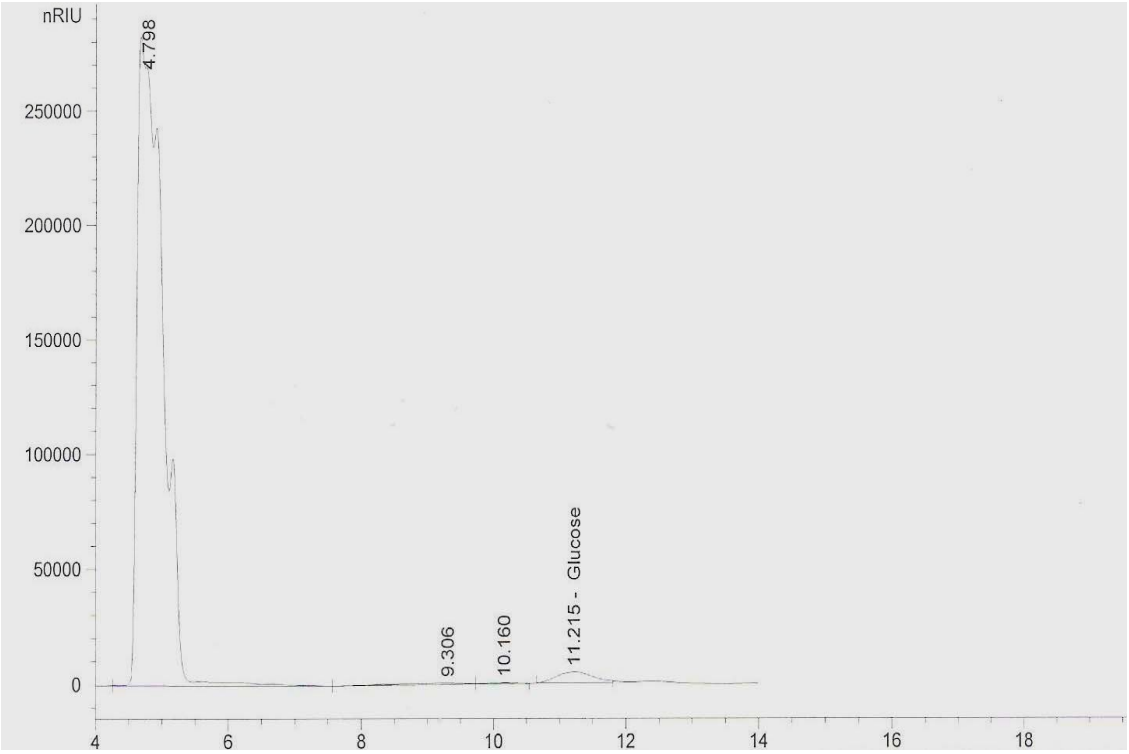
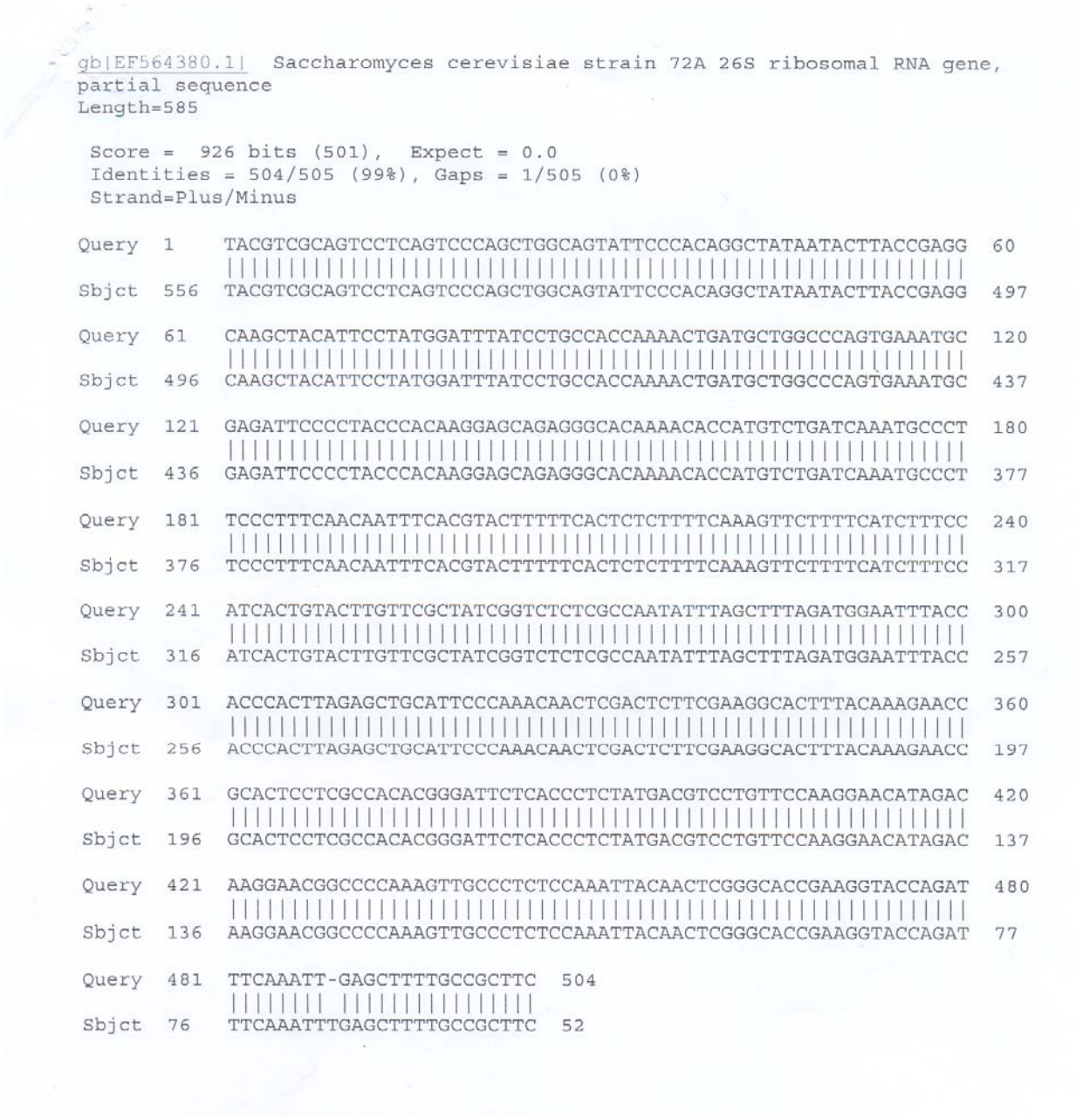


Figure 40. The HPLC-ELSD chromatogram of monosaccharide standard.

APPENDIX D

Identification of selected strain



gb|EF564380.1| Saccharomyces cerevisiae strain 72A 26S ribosomal RNA gene, partial sequence
Length=585

Score = 926 bits (501), Expect = 0.0
Identities = 504/505 (99%), Gaps = 1/505 (0%)
Strand=Plus/Minus

```
Query  1    TACGTCGCAGTCCTCAGTCCCAGCTGGCAGTATTCCCACAGGCTATAATACTTACCGAGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  TACGTCGCAGTCCTCAGTCCCAGCTGGCAGTATTCCCACAGGCTATAATACTTACCGAGG  497

Query  61   CAAGCTACATTCCTATGGATTTATCCTGCCACCAAAACTGATGCTGGCCCAGTGAAATGC  120
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Sbjct  496  CAAGCTACATTCCTATGGATTTATCCTGCCACCAAAACTGATGCTGGCCCAGTGAAATGC  437

Query  121  GAGATTCCCCTACCCACAAGGAGCAGAGGGCACAAAACACCATGTCTGATCAAATGCCCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  GAGATTCCCCTACCCACAAGGAGCAGAGGGCACAAAACACCATGTCTGATCAAATGCCCT  377

Query  181  TCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  TCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCC  317

Query  241  ATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACC  300
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Query  301  ACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCGAAGGCACTTTACAAAGAACC  360
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Sbjct  196  GCACTCCTCGCCACACGGGATTCTCACCCTCTATGACGTCCTGTTCCAAGGAACATAGAC  137

Query  421  AAGGAACGGCCCCAAAGTTGCCCTCTCCAAATTACAACTCGGGCACCGAAGGTACCAGAT  480
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Sbjct  136  AAGGAACGGCCCCAAAGTTGCCCTCTCCAAATTACAACTCGGGCACCGAAGGTACCAGAT  77

Query  481  TTCAAATT-GAGCTTTTGCCGCTTC  504
            |||||||| ||||||||||||||||
Sbjct  76   TTCAAATTTGAGCTTTTGCCGCTTC  52
```

Figure 41. Identification of selected strain by 26 S rDNA.

VITAE

Name Miss Paweena Dikit

Student ID 4883010

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology,1st Class Honor)	Prince of Songkla University	2005

Scholarship Awards during Enrolment

- The office of the Higher Education Commission, Thailand under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree.
- Development of excellency in Agro-Industry fellowship

List of Publication and Presentations

Publications

Maneerat, S. and Dikit, P. 2007. Characterization of cell-associated bioemulsifier from *Myroides* sp. SM1, a marine bacterium. Songklanakarin J. Sci. Technol. 29: 769-779.

Dikit, P., Maneerat, S. and H-Kittikun, A. 2010. Mannoprotein from spent yeast obtained from Thai traditional liquor distillation: extraction and characterization. J. Food Process Eng. Accepted.

Dikit, P., Maneerat, S., Musikasang, H. and H-Kittikun, A. 2010. Bioemulsifier from isolated yeast obtained from Thai traditional liquor distillation: extraction and characterization. ScienceAsia. Accepted.

Dikit, P., Methacanon, P., Visessanguan, W., H-Kittikun, A. and Maneerat, S. 2010. Characterization of an unexpected bioemulsifier from spent yeast obtained from Thai traditional liquor distillation. Int. J. Biol. Macromol. Accepted.

Dikit, P., Maneerat, S. and H-Kittikun, A. 2010. Encapsulation of probiotic *Lactobacillus plantarum* D6SM3 with bioemulsifier from spent yeast as emulsifier in emulsion technique and evaluation of survival in simulated gastrointestinal conditions. Biotechnol. Bioprocess Eng. Submitted.

Presentations

Dikit, P., H-Kittikun, A. and Maneerat, S. 2007. Extraction and purification of mannoprotein from spent yeast obtained from traditional liquor distillation. The 7th National Graduate Research Conference. Prince of Songkla University, Surat Thani Campus, Surat Thani, Thailand. 4-5 April 2007. pp. 108.

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